

*Investigating the role of the endogenous cannabinoid system in  
emotional modulation of pain: neurochemical and molecular  
mechanisms*

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### **Author's Declaration**

I declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. The work is original, except where indicated by the special reference in the text, and no part of the thesis has been submitted for any other academic award. Any views expressed in the thesis are those of the author.

Signed:

Date:

## Abstract

Pain represents a major unmet clinical need and affects a large number of individuals worldwide. The modulation of pain by cognitive and emotional factors is now widely recognised. Increased understanding of the endogenous mechanisms of pain modulation could lead to new or improved pain therapies. While evidence suggests that intense fear induces a potent form of endogenous analgesia termed fear-conditioned analgesia (FCA) which is adaptive and evolutionarily preserved, more sustained but less intense stress/anxiety is often associated with enhanced pain termed anxiety-related hyperalgesia (ARH). Evidence exists for a role of the endogenous cannabinoid (endocannabinoid) system in FCA; however, its role in ARH is unknown. Activation of the signal transduction molecule extracellular signal regulated kinase (Erk) and plasticity-related genes *zif268* and *sgk1* plays a role in pain, fear and CB<sub>1</sub> receptor-mediated events. The aims of the work presented in this thesis are (1) to further examine the role of the endocannabinoid system in key neural substrates in FCA and investigate molecular mechanisms underpinning endocannabinoid-mediated FCA and (2) to characterize a genetic model of ARH using two rat strains which can then be used to investigate the role of the endocannabinoid system in ARH.

FCA was modeled by combining the formalin test of persistent pain with classical Pavlovian fear conditioning in male Lister-Hooded rats. Rats received 10 footshocks followed 23.5 hrs later by an intra-right hind paw injection of formalin (2.5%) and then were re-exposed to the contextually aversive footshock arena 30min later. In the studies performed, behaviours were recorded for 3min, 15min or 30min following arena exposure. In some of these experiments, rats were implanted with cannulae into the right dorsolateral periaqueductal grey (dlPAG) approximately one week before testing and then received intracerebral injections of the fatty acid amide hydrolase (FAAH) inhibitor, URB597, or the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, 15min prior to re-exposure to the context. In other experiments, rats received systemic (intraperitoneal) injection of the CB<sub>1</sub> receptor antagonist, AM251, 30min prior to re-exposure to the context. Post-mortem analysis involved measurement of endocannabinoids and N-acylethanolamines (NAEs) using liquid chromatography coupled with tandem mass spectrophotometry (LC/MS-MS), measurement of Erk activation using western immunoblotting or measurement of the expression of *zif268* and *sgk1* mRNA in discrete brain regions or dorsal horn of the spinal cord (DHSC) by RT-qPCR. Additional work consisted of characterising a model of ARH by comparing nociceptive responding in Sprague-Dawley (SD) and Wistar Kyoto (WKY) rats, two strains with different baseline emotionality. Anxiety-related behaviour in the open field and elevated plus maze tests and nociceptive behaviour in the hot plate and the formalin tests were assessed and compared in the two strains. In one of these experiments, rats received systemic (intraperitoneal) injection of URB597 or AM251 60min and 30min prior to the behavioural tests, respectively. Post mortem work included measurement of endocannabinoids and NAEs using LC/MS-MS, measurement of the expression of CB<sub>1</sub>, FAAH, MAGL, *zif268* and *sgk1* mRNA in discrete brain regions or DHSC.

The results demonstrated a differential response of endocannabinoids and related lipids during exposure to conditioned stress, noxious inflammatory stimulus or during expression of FCA in discrete brain regions in rats including the PAG, BLA, hippocampus, insular cortex, PFC and RVM. FCA was accompanied by a strong trend towards increased levels of pErk1/2 in the right dlPAG and increased pErk1 in the right BLA. The present study demonstrated that direct administration of the CB<sub>1</sub> receptor antagonist, rimonabant, into the right dlPAG prevented FCA. In addition, intra-dlPAG URB597 was antinociceptive and showed a strong trend to enhance FCA. FCA was accompanied by attenuation of the formalin-evoked increase in the expression of *zif268* in the ipsilateral DHSC. Pharmacological blockade of the CB<sub>1</sub> receptor using systemic administration of AM251 attenuated FCA and prevented the fear-induced suppression of *zif268* expression in the ipsilateral DHSC in formalin-treated rats.

WKY rats exhibited enhanced anxiety-related behaviour and showed enhanced nociceptive responding to acute and persistent noxious stimuli compared to SD, confirming ARH. The two rat strains differed with respect to baseline levels of endocannabinoids, NAEs and expression of *CB<sub>1</sub>*, *FAAH* and *MAGL* mRNA in discrete brain regions. Moreover, systemic AM251 enhanced and URB597 attenuated ARH in WKY rats.

The data provide further evidence for an important physiological role of the endocannabinoid system within the BLA-PAG-RVM pathway in FCA, conditioned fear and pain. These data provide strong evidence for *zif268* expression in the DHSC as a molecular correlate of endocannabinoid-mediated FCA. The work presented here also suggests that alterations in central endocannabinoid function may, at least in part, underlie ARH. These results enhance our understanding of the fundamental physiology of pain and fear/anxiety and facilitate the development of new therapeutic approaches to the treatment of pain- and anxiety-related disorders and their co-morbidity.

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## List of Abbreviations

- 2-AG - 2-arachidonoylglycerol  
5-HIAA- 5-hydroxyindole-3-acetic acid  
5-HT- serotonin  
AA-5-HT- arachidonoyl serotonin  
ABHD6-  $\alpha/\beta$ -hydrolase 6  
ABHD12-  $\alpha/\beta$ -hydrolase 12  
ACC- anterior cingulate cortex  
ACEA- arachidonyl-2-chloro ethylamine  
ACTH- adrenocorticotropic hormone  
AEA- anandamide  
ANOVA- analysis of variance  
AP- anteroposterior  
ARH- anxiety-related hyperalgesia  
BLA- Basolateral amygdala  
cAMP- cyclic adenosine monophosphate  
CB<sub>1</sub>- cannabinoid receptor 1  
CCI- chronic constriction injury  
CCWS- continuous cold water swim  
CFA- complete Freund's adjuvant  
CPS- composite pain scoring  
CRF- corticotrophin-releasing factor  
CRF<sub>1</sub>-CRF receptors, subtype 1  
CRF<sub>2</sub>- CRF receptors, subtype 2  
CS- conditioned stimulus  
DA- dopamine  
DAG- diacylglycerol  
DGL- diacylglycerol lipase  
dHipp-dorsal hippocampus  
DHSC- dorsal horn of the spinal cord  
dlPAG- dorsolateral periaqueductal grey  
DMSO- dimethyl sulfoxide  
DNIC- diffuse noxious inhibitory control  
DOPAC-3,4-dihydroxyphenylacetic acid  
DV-dorsoventral  
EPM- elevated plus maze  
Erk1/2- extracellular signal regulated kinase1/2  
FAAH- fatty acid amide hydrolase  
FCA- fear-conditioned analgesia  
FM- fibromyalgia  
GABA- gamma Aminobutyric acid  
GAD- generalized anxiety disorders  
GAPDH- glyceraldehyde 3-phosphate dehydrogenase  
GIRK- G protein regulated inward rectifying channels  
HA- high analgesia  
HP- hot plate  
HPA- hypothalamic-pituitary-adrenal  
HPLC -high pressure liquid chromatography  
HVA- homovanillic acid

IBS- irritable bowel syndrome  
ICWS- intermittent cold water swim  
ip- intra-peritoneal  
IS- internal standard  
iv- intra-venous  
JNK1&2- c-Jun N-terminal kinase 1&2  
LA- low analgesia  
LC-MS/MS-liquid chromatography coupled to tandem mass spectrometry  
LE- Long Evans  
IPAG- lateral periaqueductal grey  
LPS- lipopolysaccharide  
LSD- least significant difference  
LTP- long term potentiation  
MAGL- monoacylglycerol lipase  
MAPK- mitogen activated protein kinase  
mGluR- metabotropic glutamate receptors  
ML- mediolateral  
mPFC-medial prefrontal cortex  
MRM-multiple reaction monitoring  
m/z- mass-to-charge  
NADA- N-arachidonoyl-dopamine  
NAE- N-acylethanolamines;  
NAPE- *N*-arachidonoyl-phosphatidyl-ethanolamine  
NAPE-PLD- NAPE-phospholipase D  
NAT- N-acyltransferase  
NK- neurokinin  
NMDA- N-Methyl-D-Aspartic acid  
OA- open arm  
MA- monoiodoacetate  
OCD- obsessive-compulsive disorder  
OEA- *N*-oleoylethanolamide  
OF- open-field  
PAG- periaqueductal gray  
PBN- parabrachial nucleus  
PD- panic disorder  
PEA- *N*-palmitoylethanolamide  
pErk1/2- phosphorylated extracellular signal regulated kinase1/2  
PFC- prefrontal cortex  
PH- peak heights  
PIP<sub>2</sub>- phosphoinositol bisphosphate  
PKA-protein kinase A  
PLC- phospholipase C  
PNL- peripheral nerve ligation  
po- per os (oral)  
PTSD- post-traumatic stress disorder  
RRF- relative retention factor  
RT-qPCR- real time quantitative polymerase chain reaction  
RVM- rostroventromedial medulla  
SD- Sprague Dawley  
sc- subcutaneous

SCI- spinal cord injury  
SEM- standard error of the mean  
sgk1- serum- and glucocorticoid-inducible kinase-1  
SH- Syrian Hamster  
SIA- stress-induced analgesia  
SIH- stress-induced hyperalgesia  
SNL- spinal nerve ligation  
STZ- streptozotocin  
TF- tail flick  
THC-  $\Delta^9$ -tetrahydrocannabinol  
TRPV1- transient receptor potential vanilloid subtype 1  
US- unconditioned stimulus  
vHipp- ventral hippocampus  
vlPAG- ventrolateral periaqueductal grey  
VOC- voltage operated channels  
WKY- Wistar Kyoto

## List of publications and abstracts

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Rea, K, **Olango WM**, Roche, M, Harhen, B, Kerr, D, Galligan R, Fitzgerald, S, Moore M, Finn, DP (2011). Evidence for a role of GABAergic and glutamatergic signalling in the rat basolateral amygdala in endocannabinoid-mediated fear-conditioned analgesia. *6<sup>th</sup> conference of cannabinoids in medicine. The International Association for Cannabinoid Medicines (IACM) September 2011.*

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**Olango WM**, Géranton SM, Moriarty O, Roche M, Hunt SP, Finn DP (2010). Identification of genes expressed during conditioned fear, pain and fear conditioned analgesia. 31<sup>st</sup> World Congress on Stress and Anxiety Research, Galway, Ireland, August 2010.

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## **Oral presentations**

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**Olango WM**, Roche M, Finn DP (2009). The role of the endocannabinoid system in the rat dorsolateral periaqueductal grey in fear-conditioned analgesia. *3<sup>rd</sup> Neuroscience Ireland Conference*, Trinity College Dublin, Ireland; Sept, 2009

## ***Chapter 1: General introduction***

### ***1.1 Pain***

The International Association for the Study of Pain defines pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage’(IASP Task Force on Taxonomy, 1994). Pain is a major unmet clinical need posing a great challenge to medical professionals. The physiological function of pain is to warn of tissue damage and promote survival. This role of pain is exemplified in the fact that people with congenital indifference/insensitivity to pain often die early from the effects of undetected injuries/diseases that are signaled by pain (Nagasako *et al.*, 2003). However, when pain does not subside and, instead, becomes chronic it loses its adaptive qualities and causes considerable distress and impairment of social and occupational functioning.

Determining the prevalence of chronic pain in the general population has been challenging due to variations between populations sampled, data collection methods and the criteria used to define chronic pain (Turk, 2002). Chronic pain can be defined as pain persisting for 3–6 months or longer and may be neuropathic, inflammatory or idiopathic in nature (Aguggia, 2003). A systematic review reported a mean prevalence of chronic pain of 35.5% with the range varying from 11.5 to 55.2% in a predominantly Anglo-Saxon environment (IASP, 2003). Approximately 10% of North Americans report having experienced chronic idiopathic pain in the past 12 months (Verhaak *et al.*, 1998) and one in five Europeans suffer from chronic pain, a third of which describe their pain as severe (Breivik *et al.*, 2006). Economically, pain has a direct cost of about \$100 billion annually in the US and twice that amount when one includes indirect costs due to lost productivity (McCarberg *et al.*, 2006). Hence, chronic pain is among the most disabling and costly medical conditions.

#### ***1.1.1 Clinical conditions associated with chronic pain and their management***

The most common locations of pain are the back, joint, head or neck and leg and the most common causes of pain are arthritis (osteoarthritis and rheumatoid arthritis), herniated discs, degeneration or fractures of spine, trauma or surgery and headaches (Breivik *et al.*, 2006). Clinically, pain is classified based on its

etiology. Pain that results from intrinsic damage to the neuronal pathways that normally transmits information about painful stimuli either in the periphery or centrally is termed neuropathic pain. Neuropathic pain is a feature of systemic illnesses (diabetes mellitus), infectious diseases (HIV, herpes simplex), neurological diseases (multiple sclerosis, Parkinson's disease), cancer and drug toxicity (anti tuberculosis, anti retroviral, anti cancer chemotherapy). The other common form of maladaptive pain is inflammatory pain, the majority of which involves joint inflammation-arthritis (Scutellari *et al.*, 1998; Petersson, 1996). Tissue trauma following surgery can also result in post-operative pain which occurs in up to 10% of all operations (Cousins and Power, 2003). Other painful clinical conditions include musculoskeletal pain and pain syndromes such as fibromyalgia.

Management of pain has a beneficial effect in clinical endpoints such as quality of life and mortality (Ballantyne *et al.*, 1993). As a result, the physiological benefit of aggressive pain control is compelling. The use of analgesic drugs in the clinical setting has reduced morbidity and improved satisfaction and quality of life for patients and their families. Currently available pharmacological treatments include non-steroidal anti-inflammatory drugs, opioids, anticonvulsants, anti-depressants, local anaesthetics and cannabinoids. However, the current arsenal of drugs for pain management suffer from a lack of efficacy in some patients or pain disorders (with 40% of chronic pain being treated unsatisfactorily (Breivik *et al.*, 2006)) and can be associated with serious adverse side effects including addiction, gastrointestinal upset, impaired motor coordination, impaired cognitive performance and sedation. Thus, there is a need for continued investigation of the neurobiological mechanisms of pain and its modulation with a view to identifying new potential therapeutic targets and development of superior analgesics.

### ***1.1.2 Neural pathways mediating and modulating pain***

Upon exposure to noxious mechanical, thermal or chemical stimuli, sensory information is relayed to the dorsal horn of the spinal cord (DHSC) via the primary afferent neurons. These sensory neurons, which are generally classified into two types (A-delta and C fibres) based on their diameter, structure and

conduction velocity, make synaptic connection with the secondary sensory neurons (i.e the neurons that project to brain) in the DHSC. Under normal circumstances, myelinated A-delta fibres elicit a rapid sharp type of pain, whereas unmyelinated C fibres evoke a late, dull pain that lasts longer (see review Millan, 1999). These sensory fibres are sensitized by substances such as the kinins, nitric oxide, histamine, prostanoids, adenosine and serotonin (Braszko *et al.*, 1975; Fox *et al.*, 1974; Gaponiuk, 1968; Hutter *et al.*, 1988; Rosenthal, 1949). The primary afferent neurons relay the sensory information to distinct regions within the ten laminae of the dorsal horn for the reception, processing and rostral transmission of nociceptive information (Molander *et al.*, 1986; Swett *et al.*, 1985). Nociceptive information is then conducted via second order sensory neurons to the brain (Willis, 1985a; Willis, 1985b) via the ascending pain pathways.

#### **1.1.2.1 Ascending pain pathways**

Among the pathways that project directly to higher, cerebral structures are the spinothalamic pathways, the spinoparabrachial pathway, the spinomesencephalic, the spinohypothalamic and the spinoreticular pathways (for review see Millan, 1999). Most of second order neurons in these ascending pathways traverse the midline resulting in a contralateral projection to their cerebral targets.

The ventral spinothalamic pathway densely innervates thalamic nuclei and is vital in the sensory-discriminative aspects of pain, allowing for the perception of noxious stimuli with regard to their intensity, location, duration, temporal pattern and quality. This pathway, subsequently, synapses with third-order neurons that terminate in the postcentral gyrus of the cortex (Hunt *et al.*, 1985; Willis *et al.*, 1997). Neurons in the spinoparabrachial pathway project to the parabrachial area, where they synapse with third-order neurons that terminate in the ventral medial nucleus of the hypothalamus and the central nucleus of the amygdala carrying information to areas of the brain that are concerned with cognitive-affective dimension of the pain experience. This pathway allows the appreciation of the relationship between pain and emotion and the capacity to cope with pain (Derbyshire *et al.*, 1997; Millan, 1990). In addition, the

spinomesencephalic, the spinoreticular, and the spinohypothalamic pathways all play important roles in the affective dimension of pain (for review see Millan, 1999).

Pain research using techniques such as electrophysiology, anatomical lesion, metabolic and cerebral blood flow imaging techniques have improved the understanding of pain processing in the brain. Cortical regions that respond to noxious stimuli include the inferior, anterior and post-central gyrus of parietal cortex, the insular cortex, the anterior cingulate cortex and the medial prefrontal cortex (Casey *et al.*, 1994; Svensson *et al.*, 1997). The thalamus has long been regarded as the key relay structure for the supraspinal receipt, integration and further transfer of nociceptive information to the cortex (Guilbaud *et al.*, 1990) and is involved in both sensory discriminative as well as the affective-cognitive aspects of pain through its distinct thalamocortical connections (Derbyshire *et al.*, 1997). Other direct and indirect target brain regions of ascending nociceptive inputs from the DHSC include the periaqueductal gray (PAG) and parabrachial nucleus (PBN), the amygdala, striatum, hypothalamus, nucleus accumbens and hippocampus. Some of these brain regions are part of the limbic system and are vital for motivational and cognitive aspects of pain. Thus, there are multiple complex and highly interactive direct and indirect, connections of different brain regions via multiple ascending pathways. These supraspinal targets themselves are extensively interlinked and most also participate in the descending modulation of pain (see section 1.1.2.2 below).

A number of neurochemicals synthesized in and released from the primary afferent neurons are believed to participate in transmission and modulation of nociceptive information. Studies suggest that transmission of nociceptive information via the ascending pathways mainly involves glutamatergic neurotransmission, and other excitatory neuropeptides, including substance P and cholecystokinin (Jatsu Azkue *et al.*, 1997). Others include calcitonin gene related peptide, adenosine triphosphate, nitric oxide, prostaglandins and neurotrophins (Yaksh and Malmberg, 1994).

### **1.1.2.2 Descending pain pathways**

#### **1.1.2.2.1 Endogenous control of nociception: Descending Inhibition**

Over 30 years ago, Melzack and Wall, 1965 suggested that nociceptive information delivered to the DHSC from the periphery is not automatically transferred to higher centres. Rather, according to their ‘Gate control theory’, neural processes integrated at the DHSC, profoundly modify pain-signalling prior to their dispatch to supraspinal centres. Later in 1969 electrical stimulation of the PAG was shown to induce analgesia (Reynolds, 1969). Subsequent work revealed that chemical (generally glutamate) and electrical stimulation of numerous cerebral structures can modulate nociception via pathways descending directly to the DHSC (Fields *et al.*, 1991; Willis, 1988). It is now well established that nociception resulting from the activation of ascending pathways can be modified and potentially reduced by supraspinal structures in a process known as descending inhibition. Descending pathways originating or receiving inputs from the brainstem and other cerebral structures play key roles in the modulation and integration of nociceptive transmission in the DHSC (Beitz, 1982; Hopkins *et al.*, 1978). Descending pathways modulate nociception generally by modulating the release of neurotransmitters from neurons in the DHSC including the terminals of primary afferent neurons, secondary sensory neurons and interneurons (Millan, 1999).

Supraspinal regions giving rise to pathways descending directly to the DHSC include different nuclei of the hypothalamus, PBN, nucleus tractus solitarius rostroventromedial medulla (RVM), dorsal reticular nucleus of the medulla, the PAG and the cerebral cortex (insula, cingulate, frontal and parietal) (Millan, 1999; Willis *et al.*, 1997). The central nucleus of the amygdala, which receives input from the basolateral amygdala (Neugebauer *et al.*, 2004), is the major output nucleus for projections to the PAG (Hopkins *et al.*, 1978; Oka *et al.*, 2008) and regulate the modulation of the descending inhibitory pain pathway by the PAG (Behbehani, 1995). In the RVM, the so-called OFF-cell neurons suppress pain and are involved in the mechanisms modulating descending inhibition (Heinricher *et al.*, 2009). Thus, the amygdala, PAG and RVM comprise major sites for the polysynaptic activation of descending, inhibitory pathways projecting to the DHSC (Basbaum *et al.*, 1984b).

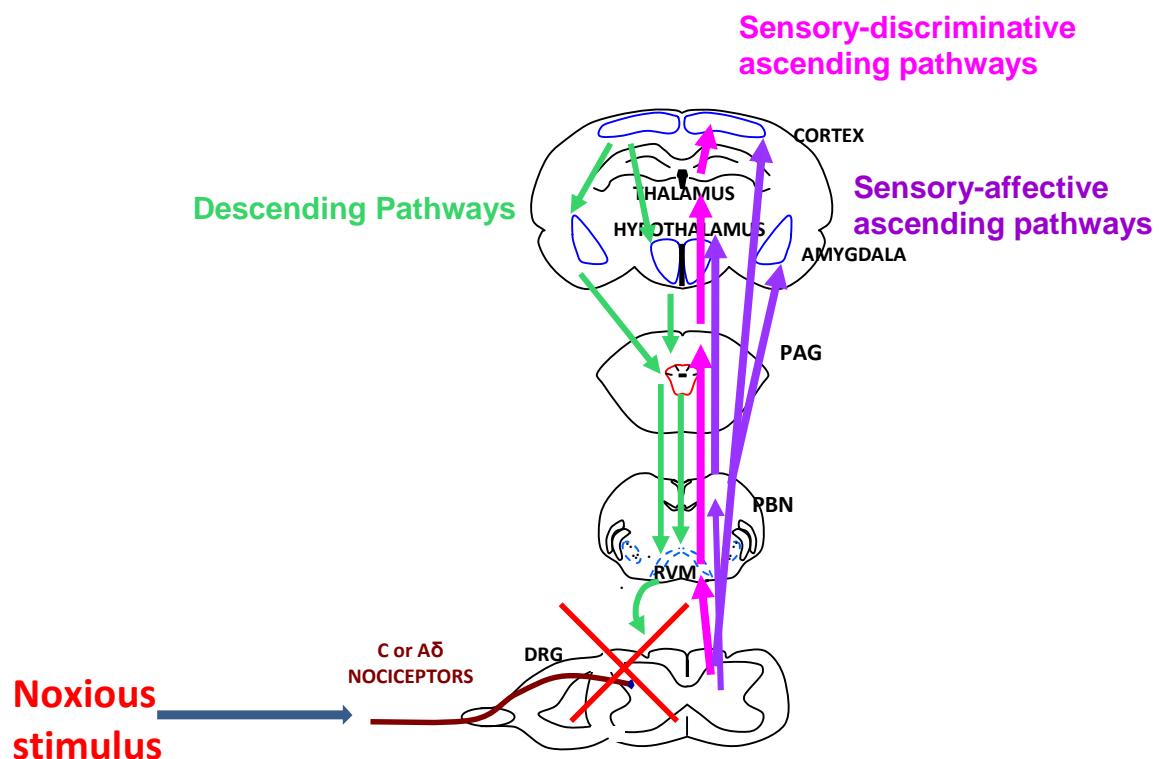
In terms of neurotransmitters involved, serotonergic, noradrenergic and dopaminergic networks comprise major components of these descending mechanisms (Fields *et al.*, 1991; Willis, 1988). Acetylcholinergic, enkephalinergic and GABAergic mechanisms of descending inhibition have also been implicated (Millan, 2002). Furthermore, opioid and cannabinoid mediated descending analgesia has been described (see section 4).

#### ***1.1.2.2 Endogenous control of nociception: Descending Facilitation***

In addition to descending inhibition, there are also descending facilitatory mechanisms that enhance rather than impede the ascending transmission of nociceptive information from the DHSC. However, there is no absolute anatomical separation of substrates subserving these processes and activation of a single supraspinal structure may, via contrasting mechanisms, trigger both descending inhibition and descending facilitation (Watkins *et al.*, 1994). The existence of multiple receptors for individual neurotransmitters which differentially modify neuronal activity could partially explain the divergent effect of descending pathways on nociception. For example, of the 15 serotonin receptor subtypes, some are excitatory and others inhibitory (Boess *et al.*, 1994). Thus a single transmitter via divergent actions expressed through different receptor types, can concomitantly promote and suppress nociceptive transmission in the DHSC.

A balance between mechanisms of descending inhibition and descending facilitation is believed to exist. Under resting conditions, descending inhibition dampens excessive sensitivity to noxious stimuli (Laird *et al.*, 1990). This equilibrium can be modified under physiological or pathological conditions. For example, during exposure to threatening conditions, descending inhibition predominates to ensure that pain doesn't compromise performance (such as escape from the threat) (Bolles *et al.*, 1980), whereas, following its cessation, descending facilitation is enhanced, normalizing nociceptive responsivity to prevent further injury from painful stimuli (Watkins *et al.*, 1994). Moreover, mechanisms of descending facilitation can be involved in pathological pain such as chronic inflammatory, neuropathic or visceral pain (Pertovaara, 1998) contributing to the hyperalgesia (increased pain from a stimulus that normally

provokes pain), and/or allodynia (pain due to a stimulus that does not normally provoke pain) which characterizes these conditions.



**Fig. 1.1** Ascending and descending pain pathways; The ventral spinothalamic pathway densely innervates thalamic nuclei subsequently synapsing with third-order neurons that terminate in the postcentral gyrus of the cortex and is vital in the sensory-discriminative aspects of pain. Neurons in the spinoparabrachial pathway project to the parabrachial nucleus (PBN), where they synapse with third-order neurons that terminate in the hypothalamus, amygdala and cortex and are important in cognitive-affective aspects of the pain experience. The descending inhibitory pain pathway originates in neurons in higher brain regions such as the cortex, hypothalamus, and amygdala. Neurons from these regions project on to the periaqueductal grey (PAG) and the rostroventromedial medulla (RVM) and finally to the dorsal horn of the spinal cord. Activation of this pathway may either inhibit or facilitate pain depending on the subset of neurons and receptors activated. DRG: dorsal root ganglia.

## **1.2 Fear/anxiety/stress**

The term ‘stress’ generally refers to a disturbance in the homeostasis of the organism and can be psychological or physical. Exposure to stress normally triggers a complex, multi-systemic response, the function of which is to restore homeostasis in response to the perturbation (Herman *et al.*, 2003). However, when the stress response is inadequate or excessive and prolonged, the cost of maintaining homeostasis becomes too high and might thus put an individual at risk of developing illness such as affective disorders (Chrousos *et al.*, 1992; de Kloet *et al.*, 2005). Fear, on the other hand, is a normal reaction to threatening/stressful situations and is a common occurrence in daily life. It is a crucial and adaptive component of the overall behavioral and autonomic stress response to dangerous situations which threaten to perturb homeostasis (Mineka *et al.*, 2002; Rosen *et al.*, 1998b). Fear proportional to the challenge encountered elicits an appropriate response (such as escape or avoidance) and is of fundamental importance as a survival strategy. Under normal circumstances a homeostatic balance is maintained. However, when fear becomes greater than that warranted by the situation, or begins to occur in inappropriate situations causing a shift of equilibrium, it becomes maladaptive and a fear or anxiety disorder may result (Marks, 1987). Such disturbance of mechanisms controlling the emotional state and the response to stress could be due to genetic, developmental and/or environmental factors (Heim *et al.*, 1999; Holmes, 2001).

### **1.2.1 Anxiety disorders**

Anxiety disorders are among the most prevalent mental health problems across the individual life span, a recent survey reporting a prevalence as high as 18-22% (Kessler *et al.*, 2005; Wittchen, 2002). Clinically, anxiety disorders are diagnosed when people consistently feel fear and experience abnormal sympathetic nervous system arousal (a.k.a. the fight-or-flight response) despite the fact that there is no real threat or danger to the person, and to the point that it is interfering with their day-to-day lives. According to DSMIV, anxiety disorders are classified into distinct nosological entities, namely, generalized anxiety disorders (GAD), panic disorder (PD), phobias, post-traumatic stress disorder (PTSD), and obsessive-compulsive disorder (OCD) (APA, 2000). Abnormalities in the fear circuitry of the brain is believed to underlie anxiety

disorders (LeDoux, 1996). It is thus important to understand in as much detail as possible how the fear circuitry works as this information may lead to a better understanding of how anxiety disorders arise and how they might be prevented or controlled.

There are a number of experimental tools for studying fear and anxiety and one of the simplest and most commonly used is classical Pavlovian fear conditioning where a relatively neutral stimulus (the conditioned stimulus, CS) is paired with a stressor (the unconditioned stimulus, US). This pairing results in long-lasting changes in the brain, such that the CS comes to elicit behavioral and neuroendocrine responses that are characteristically expressed in the presence of danger (LeDoux, 1996). Thus, fear conditioning paradigms are used to understand the neural mechanisms that enable acquisition, consolidation and extinction of fear.

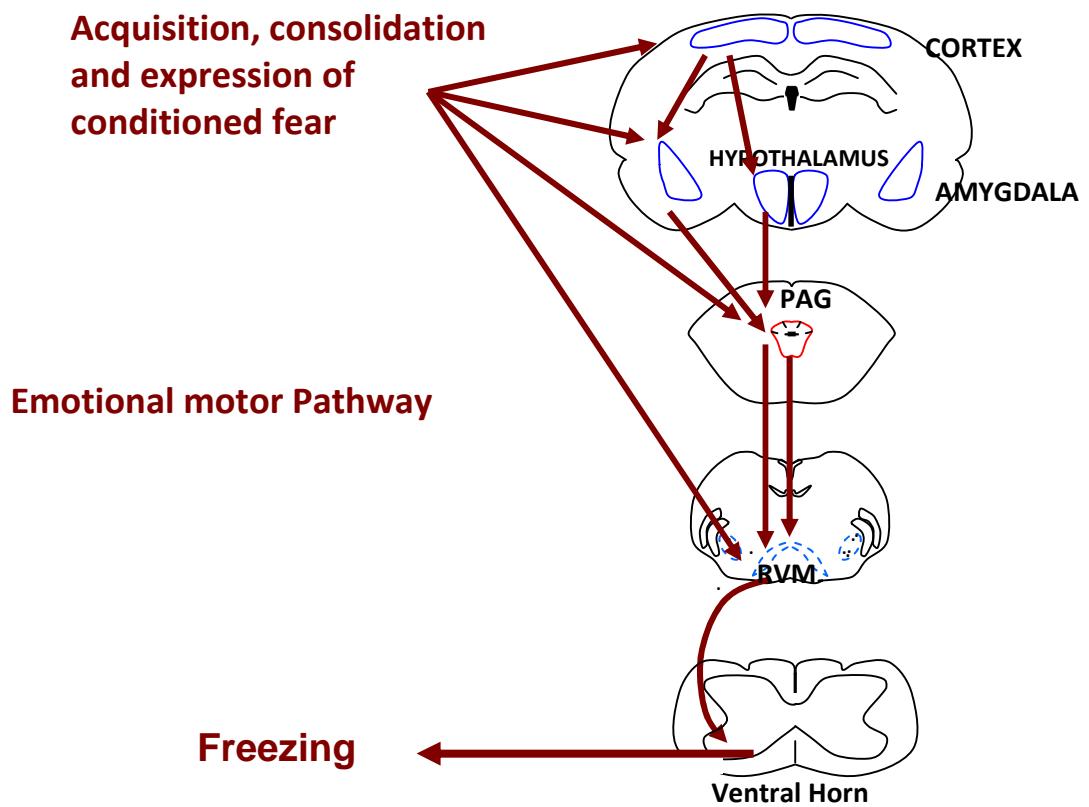
### ***1.2.1 Neurobiology of fear/anxiety***

Practically all major systems: motor, sensory, endocrine, immune, cardiovascular and, neural are involved in the response to stress and fear or anxious states (Chapman *et al.*, 2008). Similar to other higher functions, such as pain and memory, complex and diffuse networks rather than any well-defined unit, represent the experience of anxiety. The first attempt to provide a neuroanatomical and physiological explanation for the expression of emotion and fear was made by Papez 1937 (Papez, 1995) and later modified by additional work (Barili *et al.*, 1998; Gray, 1987; Gray *et al.*, 1996; Pralong *et al.*, 2002). According to these authors, the “*Circle of Papez*” consists of projections from the hippocampus to the mammillary bodies; and embraces anterior nucleus of the thalamus, cingulate cortex, amygdala, frontal cortex and other subcortical nuclei, such as the septum and nucleus accumbens. Nearly all cortical regions (insular, orbital, entorhinal, temporal, association, frontal, pre-frontal, cingulate and parietal) have now been shown to play significant, yet contrasting/unique, roles in the modulation of fear and stress (Bechara *et al.*, 2000; Groenewegen *et al.*, 2000; Hurley *et al.*, 1991; Pralong *et al.*, 2002). The hippocampus (Phillips *et al.*, 1992) and medial prefrontal cortex (Morgan and

LeDoux 1995) in particular project to the amygdala, and these 3 regions are thought to play key roles in various aspects of fear conditioning.

As is the case in pain, the amygdala, PAG and RVM are key components of the defensive fear response. The amygdala is currently considered an important forebrain region involved in several forms of aversive learning. It is involved in modulating the response to fear in animals and humans, and is a major site of action for anxiolytic agents (Davis *et al.*, 1999; LeDoux, 2000; Swanson *et al.*, 1998; Weidenfeld *et al.*, 2002). The amygdala has been implicated in the induction, processing and extinction of conditioned fear (Fanselow, 1994; Davis *et al.*, 1994; LeDoux, 2000; Marsicano *et al.*, 2002). In addition, it enjoys wide-ranging reciprocal connections with limbic, cortical and other structures implicated in the response to stress (LeDoux, 2000; Swanson *et al.*, 1998). The PAG is a mesencephalic structure involved in coordinating the defensive and aversive response to fear and stress and is another major component of the circuitry responsible for anxiety-related defense responses (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux, 1998; Schenberg *et al.*, 1990). Electrical or chemical stimulation of the PAG is aversive, both in animals and in human subjects (Lovick, 2000; Bandler *et al.*, 2000; Blanchard *et al.*, 2001; Graeff *et al.*, 2001). Along with the hypothalamus in the forebrain and the PAG in the midbrain, neurons in the RVM which includes the raphe nuclei, raphe pallidus and raphe magnus are implicated in the expression of some of the cardiac, vascular and somatic motor components of conditioned fear in what is described as the descending emotional motor system (Holstege *et al.*, 1996). Other neural substrates worth mentioning here are: the paraventricular nucleus of hypothalamus, which is vital in integrating the adrenocortical response to stress (Herman *et al.*, 2002) and the inferior colliculus which integrates aversive input and coordinates anxious and defensive behaviors (Macedo *et al.*, 2002). Hence, numerous interconnected corticolimbic structures have been implicated in the coordination and modulation of the overall response to fear and stress. Some of these regions are heterogeneous in that they incorporate functionally-distinct nuclei (Bandler *et al.*, 2000; LeDoux, 2000).

Many neurotransmitters and receptors are known to fulfill multiple roles in the modulation of anxious states, acting in contrasting or similar fashions depending on the precise cerebral circuits with which they interact, and the precise timing and conditions of their engagement (for review see Millan, 2003). Important roles for the monoaminergic, GABAergic, glutamatergic, opioidergic and cannabinergic neurotransmitter/neuromodulatory systems in the fear circuitry have been described. As discussed above, all of these systems also play key roles in mediating and modulating pain. Indeed, it is abundantly clear that there is very significant overlap in the neurochemical systems and brain regions regulating both pain and fear/anxiety/aversion. The role of these neurochemical systems in stress-pain interactions will be examined in more detail towards the end of this chapter. Since the endogenous cannabinoid system is the system of major focus for the research presented in this thesis, it will now be introduced and described in detail.



**Fig 1.2** Circuitry involved in the expression of conditioned aversive freezing. The hippocampus, cortex and amygdala process contextually aversive information. Neurons from these brain regions project to the rostroventromedial medulla (RVM) via the periaquectal grey (PAG) and finally to the ventral horn of the spinal cord to elicit a freezing response.

### **1.3 The endocannabinoid system**

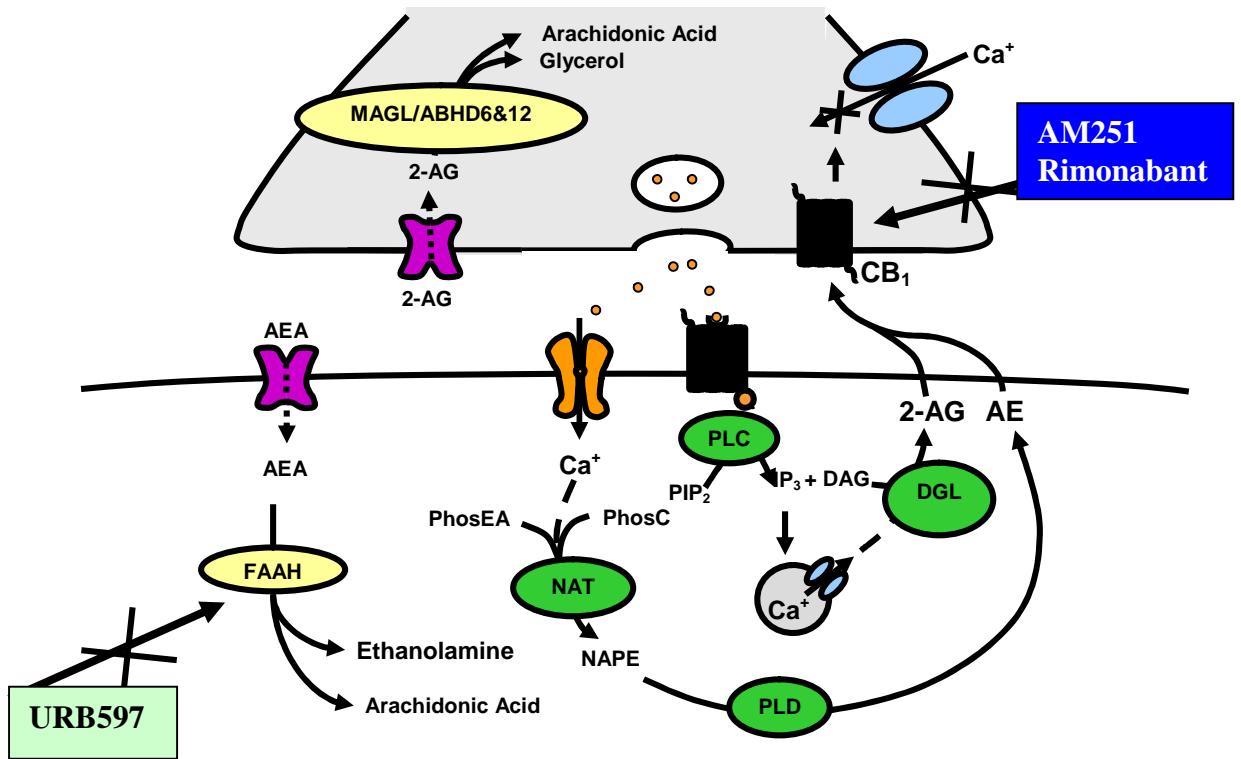
Although the medicinal properties of the plant *Cannabis sativa* have been known for millenia, it was not until the nineteenth century that its therapeutic potential was examined scientifically. After the isolation and identification of the principal psychoactive constituent of cannabis,  $\Delta^9$ -tetrahydrocannabinol (THC), in the 1960s (Mechoulam *et al.*, 1967), extensive studies have revealed the mechanisms underlying the physiological and pharmacological effects of cannabinoids. The discovery (Devane *et al.*, 1988) and cloning of the cannabinoid receptor<sub>1</sub> (CB<sub>1</sub>) in 1990 (Matsuda *et al.*, 1990 ) and cannabinoid<sub>2</sub> receptor (CB<sub>2</sub>) in 1993 (Munro *et al.*, 1993), the molecular targets mediating the effects of THC and other cannabinoids, was a significant milestone in the advancement of this topic. A year later, efforts to identify endogenous ligands led to the discovery of the first of seven endogenous cannabinoids (endocannabinoids), *N*-arachidonylethanolamide (anandamide; AEA) (Devane *et al.*, 1992). The other endocannabinoids include homo  $\gamma$  linolenyl ethanolamide and docosatetraenyl ethanolamide (Hanus *et al.*, 1993), 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), noladin ether (Hanuš *et al.*, 2001), virodhamine (Porter *et al.*, 2002) and *N*-arachidonoyl-dopamine (NADA) (Price *et al.*, 2004). Of these endocannabinoids, the most investigated to date have been AEA and 2-AG. The two cannabinoid receptors, the endogeneous ligands together with the enzymes (responsible for the synthesis and hydrolysis of these ligands (see below) and transport mechanisms constitute the endocannabinoid system. Studies using pharmacological techniques (agonists or antagonists at these receptor sites), genetic techniques (CB<sub>1/2</sub> knock out mice), autoradiography, immunohistochemistry, radioligand binding, *in-situ* hybridization, behavioural assays (the tetrad response), and others have significantly improved our understanding of this system. Today the endocannabinoid system is known to be important in a broad range of physiological processes and behaviours including mood disorders, pain and inflammation, appetite, emesis, vision, memory, immunity and the cardiovascular system (Di Marzo, 2008).

### **1.3.1 Metabolic pathways of endocannabinoids**

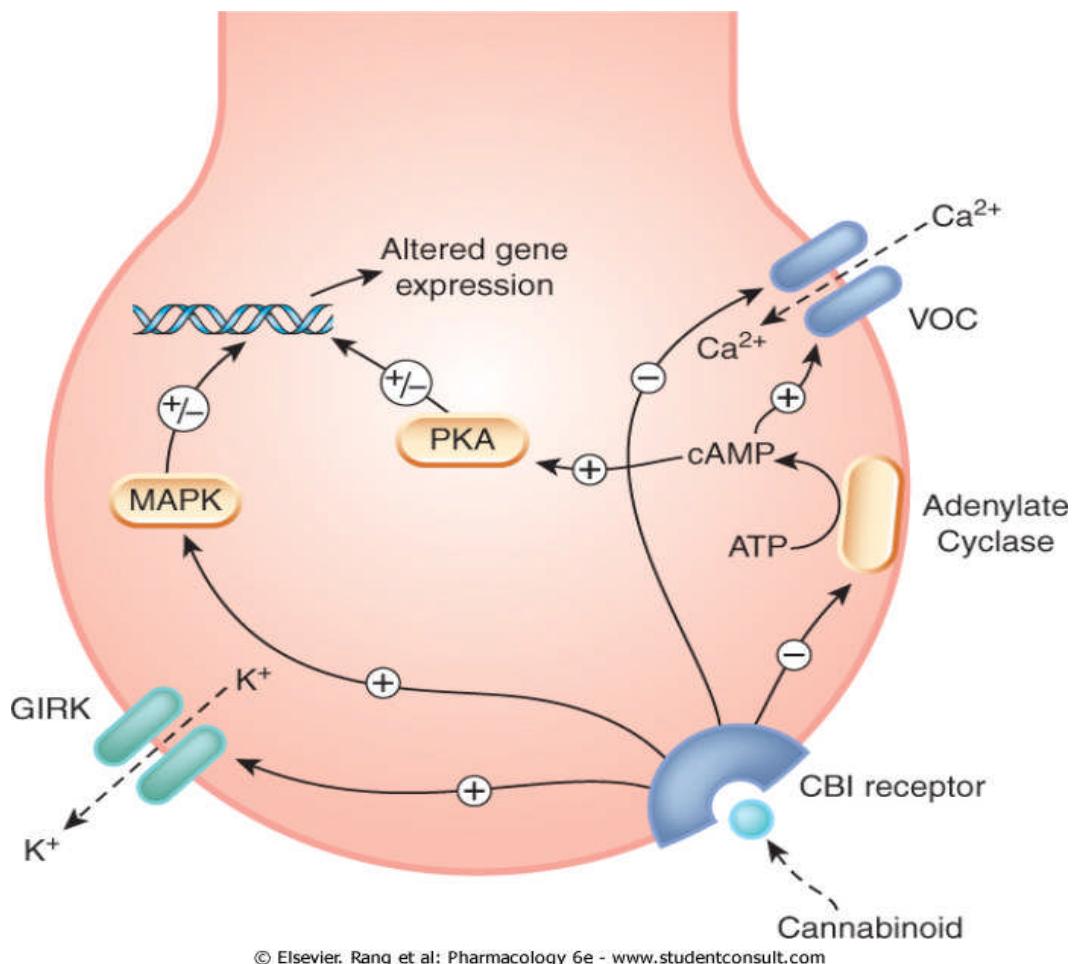
Several pathways exist for the formation and catabolism of anandamide and 2-AG. Anandamide originates from a phospholipid precursor, *N*-arachidonoyl-phosphatidyl-ethanolamine (NAPE), mainly through a pathway that is catalysed by a NAPE-selective phosphodiesterase (Di Marzo *et al.*, 2007; Liu *et al.*, 2008). Alternative enzymes proposed to be responsible for AEA biosynthesis include  $\alpha/\beta$ -hydrolase (Simon *et al.*, 2006) and phospholipase C (PLC) (Liu *et al.*, 2006b). Other fatty-acid ethanolamides that do not have significant affinity to cannabinoid receptors (a.k.a. the *N*-acylethanolamines; NAE), such as, *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA), can also be formed through these pathways (Alexander *et al.*, 2007). NAEs attracted attention as bioactive lipids for the first time in 1957 when anti-inflammatory activity of PEA was shown in guinea pigs (Kuehl *et al.*, 1957). PEA and OEA are formed from the phospholipid, NAPE by several enzymatic pathways (for review see Ahn *et al.*, 2008). The major pathway is catalyzed by a membrane-associated NAPE-phospholipase D (NAPE-PLD) generating the acylethanolamide and phosphatidic acid (Leung *et al.*, 2006; Okamoto *et al.*, 2004). An alternative enzymatic pathway includes de-acylation of the O-acylated fatty acids of NAPE followed by a hydrolysis of the generated glycerophospho-*N*-acylethanolamine by a phosphodiesterase (Simon *et al.*, 2006; Simon *et al.*, 2008).

2-AG is synthesized almost exclusively from phospholipid precursors by PLC and diacylglycerol lipase (Schlosburg *et al.*, 2009). Compared with 2-AG, AEA has the higher affinity for both CB<sub>1/2</sub> receptors, however, 2-AG has higher efficacy than AEA at both receptors (Di Marzo, 2008; Howlett *et al.*, 2000). AEA appears to be a partial agonist for CB<sub>1</sub> receptor, while 2-AG is a full agonist for both CB<sub>1</sub> and CB<sub>2</sub> (Sugiura *et al.*, 1999; Sugiura *et al.*, 2000). Endocannabinoids are generally considered to be released on demand from cells immediately after biosynthesis, as no evidence exists for their storage in secretory vesicles (Di Marzo *et al.*, 2007). The process involving transport of endocannabinoids into neural cells for degradation is controversial. The lack of molecular evidence for the presence of an AEA “membrane transporter” has generated a debate on this subject. Although this putative “transporter” has yet

to be cloned, indirect biochemical and pharmacological evidence suggests its existence. Evidence suggests that the mechanism is most likely transporter-mediated facilitated diffusion (Beltramo *et al.*, 1997; Ligresti *et al.*, 2010; Ligresti *et al.*, 2004). After cellular re-uptake AEA is metabolized primarily via fatty acid amide hydrolase (FAAH) which is located in the endoplasmic reticulum of postsynaptic neurons (Cravatt *et al.*, 1996; Otrubova *et al.*, 2011; Seierstad *et al.*, 2008). FAAH is also responsible for the catabolism of non-cannabinoid NAE, including PEA and OEA (Cravatt *et al.*, 1996; Ahn *et al.*, 2008). Monoacylglycerol lipase (MAGL) located on the presynaptic terminal (Giang *et al.*, 1997) appears to play the predominant role (85%) in 2-AG degradation (Ueda, 2002). Two other enzymes,  $\alpha/\beta$ -hydrolase 6 and 12 (i.e., ABHD6 and ABHD12), as well as FAAH together contribute to the other 15% of 2-AG metabolism in the nervous system (Blankman *et al.*, 2007; Di Marzo, 2008). To date, in addition to antagonists (e.g. rimonabant, AM251; Pertwee, 2006) and agonists (e.g. HU210, WIN 55,212-2, Pertwee, 2006) at cannabinoid receptor, specific inhibitors/blockers have been developed for FAAH (URB597, OL-135 and PF-3845; Booker *et al.*, 2011), MAGL (JZL184; Pan *et al.*, 2009), and the putative endocannabinoid transporters (AM404; Ho *et al.*, 2005).



**Fig 1.3** Diagrammatical representation of an endocannabinoid synapse. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized following an increase in cytosolic calcium ( $\text{Ca}^{2+}$ ) resulting from activation of postsynaptic ion channels or G protein-coupled receptors. The activation of Gq protein-coupled receptors results in the synthesis of inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) from phosphoinositol bisphosphate (PIP<sub>2</sub>). IP<sub>3</sub> mobilizes calcium release from intracellular stores, triggering the formation of 2-AG from DAG by the enzyme diacylglycerol lipase (DGL). The activation of  $\text{Ca}^{2+}$  gating ion channels facilitates the influx of  $\text{Ca}^{2+}$ , which leads to the formation of N-arachidonoyl-phosphatidylethanolamine (NAPE) from phosphatidylethanolamine (PhosEA) and phosphatidylcholine (PhosC) via the enzyme N-acetyltransferase (NAT). NAPE is then hydrolyzed to AEA by a phospholipase D-type enzyme (NAPE-PLD). The cannabinoids are released from the postsynaptic neuron and travel retrogradely to the presynaptic membrane to activate cannabinoid receptors (e.g. cannabinoid<sub>1</sub> receptor, CB<sub>1</sub>). The activation of the CB<sub>1</sub> receptor results in inhibition of  $\text{Ca}^{2+}$  channels in the presynaptic membrane and a number of other signal transduction-mediated events, which generally result in suppression of neuronal activity and neurotransmitter release. 2-AG is catabolized to arachidonic acid and glycerol by monoacylglycerol lipase (MAGL) and  $\alpha/\beta$ -hydrolase 6 and 12 (i.e., ABHD6 and ABHD12) while fatty acid amide hydrolase (FAAH) breaks down AEA to arachidonic acid and ethanolamine. URB597 is inhibitor of FAAH and AM251 and rimonabant are antagonists/inverse agonist at CB<sub>1</sub>. (Modified from Rea *et al.*, 2007)



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**Fig 1.4** Signal transduction pathways following activation of CB<sub>1</sub> receptor; CB<sub>1</sub> receptor is negatively coupled to adenylate cyclase and Ca<sup>2+</sup> channels and positively coupled to MAPK and K<sup>+</sup> channel; GIRK, G protein regulated inward rectifying channels; MAPK, Mitogen activated protein kinase; cAMP, cyclic adenosine mono phosphate; PKA, protein kinase A; CB<sub>1</sub>, cannabinoid receptor 1; VOC, voltage operated channels (www. studentconsult.com)

### ***1.3.2 Distribution of cannabinoid receptors***

Although CB<sub>1</sub> receptors are expressed by certain non-neuronal cells and tissues, for example the adrenal gland, bone marrow, heart, lung, prostate, testis, thymus, tonsils, and spleen (Bouaboula *et al.*, 1993; Galiègue *et al.*, 1995; Kaminski *et al.*, 1992; Noe *et al.*, 2000), they are expressed widely and in high density throughout the rodent and human brain (Herkenham *et al.*, 1991; Mackie, 2005; Tsou *et al.*, 1997). Using autoradiography studies and *in-situ* hybridization, CB<sub>1</sub> receptors were found to be abundant in cerebral cortex, hippocampus, basal ganglia, and cerebellum. Lower levels were found in the amygdala, mid-brain, hypothalamus and spinal cord and were almost absent from the respiratory centers of the brainstem, consistent with the clinical observation of the low lethality of cannabis overdose (Herkenham *et al.*, 1991; Herkenham *et al.*, 1990; Robson, 2001). In addition to the CNS, CB<sub>1</sub> receptors are expressed in the nerve terminals of peripheral neurons (Hohmann *et al.*, 1999) and other peripheral tissues including the intestine (Pertwee *et al.*, 1996) and urinary bladder (Pertwee *et al.*, 1996). The CB<sub>2</sub> receptor is primarily expressed in tissues and cells of the immune system (Munro *et al.*, 1993; Parolaro, 1999). Both *in situ* hybridization studies and autoradiographic studies have demonstrated a high expression of CB<sub>2</sub> receptors in multiple lymphoid organs (Buckley *et al.*, 1997; Lynn *et al.*, 1994). CB<sub>2</sub> receptor mRNA is found in spleen, thymus, tonsils, bone marrow, pancreas, splenic macrophage/monocyte preparations and peripheral blood cells (Bouaboula *et al.*, 1993; Facci *et al.*, 1995; Galiègue *et al.*, 1995; Munro *et al.*, 1993; Bouaboula *et al.*, 1993; Condie *et al.*, 1996). These receptors are also localized on glial cells (Cabral *et al.*, 2005; Massi *et al.*, 2008) and some recent evidence also suggests that CB<sub>2</sub> receptors may be expressed in neurons (Gong *et al.*, 2006; Onaivi *et al.*, 2008., Van Sickle *et al.*, 2005). However, the function of CB<sub>2</sub> receptors in the brain has not been characterized as thoroughly as the CB<sub>1</sub> receptor.

### ***1.3.3 Cannabinoid receptor-mediated modulation of neurotransmission***

Detailed electron microscopy studies have localised CB<sub>1</sub> receptors almost exclusively on presynaptic terminals (Hájos *et al.*, 2000; Katona *et al.*, 2000). Endocannabinoids synthesized from postsynaptic neurons signal retrogradely through CB<sub>1</sub> receptors expressed presynaptically to inhibit neurotransmitter

release. In this process, unlike the conventional neurotransmitters, the endocannabinoids travel from post-synaptic neurons to a pre-synaptic neurons. Such endocannabinoid-mediated retrograde signaling was first described by Willson and colleagues and has been proposed to occur in the hippocampus, amygdala, PAG, RVM and spinal cord (Jennings *et al.*, 2001; Katona *et al.*, 2001; Lichtman *et al.*, 1996; Meng *et al.*, 1998; Wilson *et al.*, 2001; Wilson *et al.*, 2001). Using direct measurement of transmitter levels *in vivo* or *in vitro* or indirectly using electrophysiological techniques it was shown that activation of pre-synaptic CB<sub>1</sub> receptors both in the brain and in the peripheral nervous system inhibited the release of a number of different excitatory or inhibitory neurotransmitters including noradrenaline (Göbel *et al.*, 2000; Kathmann *et al.*, 1999; Schlicker *et al.*, 1997), dopamine (Cadogan *et al.*, 1997; Kathmann *et al.*, 1999), serotonin (Nakazi *et al.*, 2000), GABA (Irving *et al.*, 2000; Katona *et al.*, 2001; Ohno-Shosaku *et al.*, 2001; Takahashi *et al.*, 2000; Vaughan *et al.*, 1999) and glutamate (Auclair *et al.*, 2000; Robbe *et al.*, 2001; Vaughan *et al.*, 2000). Although the primary effect of CB<sub>1</sub> receptor agonists on neurotransmitter release seems to be inhibition, this may result in enhanced neurotransmitter release downstream. Indeed, endocannabinoids in the CNS were shown to participate in both depolarisation-induced suppression of inhibition (decrease in GABA release) and depolarisation-induced suppression of excitation (via inhibition of glutamate release) (Kreitzer *et al.*, 2001; Maejima *et al.*, 2001).

#### **1.3.4 Cannabinoid signalling and alternate targets**

The synthesis of the endocannabinoids begin with the depolarisation of the post synaptic neurons due primarily to an elevation of intracellular Ca<sup>2+</sup>, stimulation of glutamate or dopamine receptors or by blockade of K<sup>+</sup> channels (Cadas *et al.*, 1996; Di Marzo *et al.*, 1994; Giuffrida *et al.*, 1999). This in turn stimulates the on-demand synthesis and release of endocannabinoids (Ohno-Shosaku *et al.*, 2001). Activation of metabotropic glutamate receptors (mGluRs) and muscarinic receptors can also stimulate the release of endocannabinoids in a non-Ca<sup>2+</sup>-dependent manner (Kim *et al.*, 2002; Maejima *et al.*, 2001). The CB<sub>1</sub> (Matsuda *et al.*, 1990) and CB<sub>2</sub> receptors (Munro *et al.*, 1993) are both members of the family of Gi/o-protein coupled seven transmembrane receptors. Agonist

stimulation of CB<sub>1</sub> and CB<sub>2</sub> receptors activates a number of signal transduction pathways via this Gi/o family of G proteins. Both CB<sub>1</sub> and CB<sub>2</sub> receptors are negatively coupled to adenylate cyclase and positively coupled to mitogen-activated protein kinase. Cannabinoids inhibit adenylate cyclase and thus attenuate cyclic AMP production (Matsuda *et al.*, 1990 ; Slipetz *et al.*, 1995). Both CB<sub>1</sub> and CB<sub>2</sub> receptor-mediated inhibition of adenylate cyclase is sensitive to CB receptor antagonists and pertussis toxin, indicating the requirement for Gi/o proteins (Pacheco *et al.*, 1993; Pertwee, 1997; Vogel *et al.*, 1993). The subsequent damping of phosphorylation by protein kinase A is believed to modulate signalling pathways and ion channel activity. AEA and other cannabinoids (CP 55,940, Δ9-THC, HU-210) were shown to produce a concentration-related increase in the activity of mitogen-activated protein kinase (MAPK) (particularly p42/p44) in C6 glioma and primary astrocyte cultures which is attenuated by CB receptor antagonists and prevented by pertussis toxin pretreatment, pointing to an involvement of inhibitory G-proteins (Bouaboula *et al.*, 1995b; Guzman *et al.*, 1999). MAPK was also activated in immune cells possessing CB<sub>2</sub> receptors (Bouaboula *et al.*, 1996). Activation of this pathway leads to the expression of immediate early genes, phosphorylation of phosphodiesterase A<sub>2</sub>, release of arachidonic acid, and prostaglandin E<sub>2</sub> metabolism (Wartmann *et al.*, 1995). CB<sub>1</sub> and CB<sub>2</sub> activation can lead to expression of immediate early genes, as has been demonstrated for *zif268* (a gene encoding for zinc finger protein) (Bouaboula *et al.*, 1995a; Bouaboula *et al.*, 1996; Glass and Dragunow, 1995). Cannabinoid receptor agonists also evoked an increase in other immediate early genes including c-Fos (Patel *et al.*, 1998) and c-Jun N-terminal kinase (JNK1 and JNK2) in cells expressing recombinant CB<sub>1</sub> receptors (Rueda *et al.*, 2000). CB<sub>1</sub> receptors are also coupled through Gi/o proteins to certain ion channels. Several cannabinoid receptor agonists have been shown to produce a concentration-related inhibition of voltage-activated (L,N,Q,P) inward calcium currents in a pertussis toxin sensitive manner (for review see Pertwee, 1997). CB<sub>1</sub> receptors are also known to activate A-type outward potassium currents (Deadwyler *et al.*, 1993) and increase inwardly rectifying K<sup>+</sup> channels (Mackie *et al.*, 1995).

In addition to activating CB<sub>1</sub> receptors, AEA also interacts with several non-cannabinoid receptors, a good example of which is the transient receptor potential, vanilloid subtype 1 (TRPV1) channel (for review see Pertwee *et al.*, 2010). Both AEA and 2-AG were reported by some authors to interact with an orphan G-protein-coupled receptor, GPR55 (Ryberg *et al.*, 2007), and with peroxisome-proliferator-activating nuclear receptors (for review see O'Sullivan, 2007). PEA and OEA potentiate the effect of anandamide on cannabinoid receptor and/or vanilloid receptor (De Petrocellis *et al.*, 2001; Smart *et al.*, 2002) in what has been described as “entourage effect” mediated by competitive inhibition of anandamide hydrolysis by FAAH (Jonsson *et al.*, 2001). Moreover, OEA and PEA can themselves activate TRPV1 and peroxisome-proliferator-activating nuclear receptors (for review see Hansen, 2010). Thus, caution should be exercised when attributing the effects of AEA, or drugs which increase its availability, to CB<sub>1</sub> receptors, since AEA also has direct agonistic activity at the vanilloid receptor (De Petrocellis *et al.*, 2001; Di Marzo *et al.*, 2001; Ross *et al.*, 2001; Smart *et al.*, 2000).

### **1.3.5 The endocannabinoid system and modulation of pain**

Cannabinoid receptors are localized in neuroanatomical regions subserving transmission and modulation of pain signals, such as the cortex, thalamus, basolateral amygdala (BLA), PAG, the RVM and the DHSC (Herkenham *et al.*, 1991; Tsou *et al.*, 1997) suggesting that endocannabinoids play a key role in the central nervous system modulation of pain signalling. An interesting study showed that AEA is elevated in the PAG by peripheral pain stimuli and electrical stimulation of the dorsal PAG resulted in release of anandamide and rimonabant sensitive analgesia (Walker *et al.*, 1999). The CB<sub>1</sub> receptor has been shown to be upregulated in the thalamus (Siegling *et al.*, 2001) and spinal cord (Lim *et al.*, 2003) in rodent models of neuropathic pain indicating that neuropathic pain conditions can alter the state of the endocannabinoid system.

Transgenic approaches involving FAAH and CB<sub>1</sub> knockout mice have been used in conjunction with pharmacological approaches to better evaluate the role of endocannabinoids in pain modulation. Normal thermal pain sensitivity, but increased tactile sensitivity in CB<sub>1</sub> knockout mice has been reported (Ibrahim *et*

*al.*, 2003). Mutant mice lacking the CB<sub>1</sub> gene fail to show typical antinociceptive responses to prototypical cannabinoid agonists (Ledent *et al.*, 1999; Zimmer *et al.*, 1999). The transient “knock-down” of CB<sub>1</sub> receptors in the spinal cord by antisense methods augmented pain behaviour (Dogru *et al.*, 2002; Richardson *et al.*, 1998) providing evidence in favour of a tonic endocannabinoid influence over nociception in this region of the CNS. In contrast, data from CB<sub>1</sub> knockout mice showed hypoalgesic responses in both the hot plate and formalin tests, but no change in nociceptive behaviour in the tail-flick assay (Zimmer *et al.*, 1999). It is possible that the constitutive inactivation of CB<sub>1</sub> receptors leads to either compensatory changes in the nervous system that account for the unexpected pain responses of CB<sub>1</sub> knockout mice or other behavioural changes that interfere with the measurement of nociception. In addition, FAAH knockout mice, which are severely impaired in their ability to degrade anandamide having 15-fold augmented levels of endogenous anandamide in the brain, exhibit reduced pain responses in the tail-immersion, hot plate, and formalin tests (Cravatt *et al.*, 2001; Lichtman *et al.*, 2004b) in a rimonabant sensitive fashion and reduced inflammation in a dinitrobenzene sulfonic acid model of colitis (Massa *et al.*, 2004). However, FAAH knock out mice were found not to show altered thermal pain responses in the chronic constrictive injury model of neuropathic pain (Lichtman *et al.*, 2004b).

The effects of CB<sub>1</sub> receptor antagonists on pain responses have provided equivocal findings. For example, the CB<sub>1</sub> receptor antagonist rimonabant did not alter acute pain sensitivity in the tail flick test in rats (Rinaldi-Carmona *et al.*, 1994). Rimonabant also had no effect on pain sensitivity in either the tail immersion or hot plate tests in mice (Cravatt *et al.*, 2001; Lichtman *et al.*, 2004b). In contrast, rimonabant has been reported in other studies to produce hyperalgesia in the tail flick (Costa and Colleoni, 1999) and hot plate (Richardson *et al.*, 1998) tests in rats. Intra-thecal administration of rimonabant facilitate the nociceptive responses of dorsal horn neurons to acute pain stimuli (Chapman, 1999). In the formalin test of persistent pain, CB<sub>1</sub> receptor antagonists were hyperalgesic (Calignano *et al.*, 1998; Strangman *et al.*, 1998), but subsequent reports have failed to confirm these findings (Beaulieu *et al.*,

2000; Lichtman *et al.*, 2004b). Whereas hyperalgesic effects of AM251, another CB<sub>1</sub> receptor antagonist, in the formalin test were observed in rats following systemic administration (Maione *et al.*, 2007), in the majority of studies systemic AM251 did not affect nociceptive responding (Guindon *et al.*, 2007a; Liang *et al.*, 2007; Schuelert *et al.*, 2011; Hama and Sagen, 2007; Ahmed *et al.*, 2010; Maione *et al.*, 2007) in various rodent pain models.

Several studies have demonstrated robust hypoalgesic phenotypes after pharmacological disruption of FAAH by URB597 (Hasanein, 2009; Hasanein *et al.*, 2008; Jayamanne *et al.*, 2006; Kathuria *et al.*, 2003; Kinsey *et al.*, 2009; Naidu *et al.*, 2010; Russo *et al.*, 2007). Other FAAH inhibitors (such as PMSF, URB532, AA-5HT, JNJ-1661010, OL-135) have all been shown to induce antinociception in the tail flick (Campton and martin, 1997), hot plate (Kathuria *et al.*, 2003), formalin (Maione *et al.*, 2007), carrageenan (Karbarz *et al.*, 2009), chronic constriction injury (CCI) tests (Maione *et al.*, 2007) in a CB<sub>1</sub> dependent manner. In addition, inhibitors of MAGL (e.g. URB602) and endocannabinoid uptake (e.g. UCM707, AM404, VDM11) were found to be antinociceptive in a wide range of animal models including tail flick (Hasanein *et al.*, 2008), hot plate (de Lago *et al.*, 2002; de Lago *et al.*, 2004), formalin test (La Rana *et al.*, 2006), carrageenan (Comelli *et al.*, 2007), CCI (Costa *et al.*, 2006), and PSNL (Desroches *et al.*, 2008; Mitchell *et al.*, 2007). Thus, collectively these data from both genetic models and pharmacological studies with endocannabinoid system modulators suggest that endocannabinoid signalling through CB<sub>1</sub> receptors acts largely to reduce pain-related behaviour. An extensive body of literature indicates that agonists of cannabinoid receptors are antinociceptive in a wide range of animal models of pain including acute, inflammatory and neuropathic pain. An exhaustive review of all studies is beyond the scope of this thesis but for review see Finn and Chapman, 2004.

### **1.3.6 The endocannabinoid system and modulation of anxiety**

The distribution of CB<sub>1</sub> receptors in rat brain is consistent with an involvement of this system in the regulation of emotion, with high levels of CB<sub>1</sub> expression in structures such as the amygdala, hippocampus, PAG and the cortex (Hájos *et al.*, 2002; Herkenham *et al.*, 1991; Herkenham *et al.*, 1990; Katona *et al.*, 2001).

Transgenic approaches involving FAAH and CB<sub>1</sub> knockout mice have been used together with pharmacological approaches to improve our knowledge of the role of endocannabinoids in anxiety modulation. Transgenic mice lacking expression of the CB<sub>1</sub> receptor exhibit an anxiogenic profile in a number of anxiety assays including the elevated plus-maze (Haller *et al.*, 2002; Haller *et al.*, 2004; Urigüen *et al.*, 2004), open-field (Maccarrone *et al.*, 2002; Urigüen *et al.*, 2004) and the light-dark box (Maccarrone *et al.*, 2002; Urigüen *et al.*, 2004). CB<sub>1</sub> knockout mice exhibited impaired short-and long-term extinction of cue-induced conditioned-fear responding (Marsicano *et al.*, 2002) and had lower burying scores in a shock-probe burying test of active and passive avoidance (indicative of an anxiolytic behaviour in this test) (Degroot *et al.*, 2004). In another study, CB<sub>1</sub> receptor knockout mice showed increases in conditioned aversive responses (Martin *et al.*, 2002). In addition, transgenic mice deficient for FAAH showed reduced anxiety-like behaviour in the elevated plus maze and light-dark box in a CB<sub>1</sub> receptor-dependent manner (Moreira *et al.*, 2008). However, contrasting results have been obtained, with CB<sub>1</sub> knockout mice showing no anxiogenic-like response (Marsicano *et al.*, 2002) and FAAH knockout mice showing no change in anxiety-related behavior (Naidu *et al.*, 2007) in the elevated plus-maze. Overall, however, the weight of evidence supports the hypothesis that endocannabinoids through the activation of CB<sub>1</sub> receptors are implicated in the control of emotional behaviour.

Pharmacological blockade of CB<sub>1</sub> with rimonabant elicited an anxiogenic effect in the rat elevated plus-maze (Arévalo *et al.*, 2001; Navarro *et al.*, 1997) defensive withdrawal test (Navarro *et al.*, 1997) and ultrasonic vocalization test (McGregor *et al.*, 1996). Systemic administration of rimonabant resulted in impaired short-and long-term extinction of cue- or context-induced conditioned fear responding (Marsicano *et al.*, 2002, Finn *et al.*, 2004; Roche *et al.*, 2007). Another CB<sub>1</sub> receptor antagonist, AM251 increases anxiety-related behaviours in the elevated plus maze or open field tests in mice (Haller *et al.*, 2004; Patel *et al.*, 2006a; Rodgers *et al.*, 2005) and in rats (Sink *et al.*, 2010). The anxiogenic effect of AM251 was abolished in CB<sub>1</sub> receptor knockout mice (Haller *et al.*, 2004). In addition, human studies suggest that rimonabant may be associated with increased anxiety/depression (Rosenstock *et al.*, 2008; Scheen, 2008).

However, in mice, rimonabant reduced anxiety-related behaviour in the elevated plus-maze (Haller *et al.*, 2002; Rodgers *et al.*, 2003) and in the light-dark test (Akinshola *et al.*, 1999). Haller and colleague suggested a novel target for rimonabant could be responsible as this anxiolytic effect was not CB<sub>1</sub> dependent (Haller *et al.* 2002).

The fact that pharmacological blockade of CB<sub>1</sub> receptors is largely associated with anxiety implied that pharmacological enhancement of endocannabinoid signaling may be associated with an anxiolytic phenotype. Indeed, systemic administration of the FAAH inhibitors, URB597 and URB532, reduced anxiety-related behaviours in the rat elevated zero-maze and isolation-induced ultrasonic vocalization tests (Kathuria *et al.*, 2003) in a dose-and CB<sub>1</sub>-dependent manner. URB597 has also been shown to be anxiolytic in the rat elevated plus-maze and open-field tests (Hill *et al.*, 2007; Rubino *et al.*, 2008a). In addition, intra-cerebral injection of anandamide has been reported to produce anxiolytic effects (Moreira *et al.*, 2007). AM404, the FAAH and endocannabinoid re-uptake inhibitor, also exhibits a dose-dependent anxiolytic profile in the elevated plus-maze, defensive withdrawal test and ultrasonic vocalization test (Bortolato *et al.*, 2006) in a rimonabant-sensitive manner. Moreover, systemic (Chhatwal *et al.*, 2004; Pamplona *et al.*, 2008) or intra-cerebroventricular (Bitencourt *et al.*, 2008) administration of AM404 promoted extinction of fear memories. Another study demonstrated that intra-dorsolateral PAG administration of AM404 or anandamide reduces expression of contextually induced fear in rats (Resstel *et al.*, 2008) in a CB<sub>1</sub> dependent manner. These results further support the view that there is an endogenous regulation of emotional states by the endocannabinoid system. However, the effect of anandamide on anxiety appears to depend on dose with low doses tending to be anxiolytic and higher doses anxiogenic (Akinshola *et al.*, 1999; Rubino *et al.*, 2008b; Scherma *et al.*, 2008). Data from animal tests provide further evidence for these bidirectional effects of cannabinoids on anxiety. Low doses of the cannabinoid receptor agonists, nabilone (Onaivi *et al.*, 1990), CP 55,940 (Marco *et al.*, 2004) and THC (Berrendero *et al.*, 2002) induced anxiolytic-like effects in the elevated plus-maze and light-dark crossing tests and high doses of the cannabinoid agonist HU-210 produced anxiogenic-like responses in the defensive withdrawal test

(Rodriguez de Fonseca *et al.*, 1996) and enhanced emotional responding to tactile stimulation (Giuliani *et al.*, 2000). Effects of anandamide, or drugs which increase its availability on anxiety should be interpreted cautiously as its effect depend on factors such as doses employed and the presence of alternate targets (e.g. TRPV1). Thus, further work is necessary, data from both genetic models and pharmacological studies with endocannabinoid system modulators suggest that endocannabinoid signaling through CB<sub>1</sub> receptors acts largely to reduce anxiety-related behaviour.

#### **1.4 Emotional modulation of pain**

It is now widely acknowledged that the intensity and severity of perceived pain does not necessarily correlate linearly with the degree of tissue damage/injury/inflammation or the intensity of nociceptive activity. In this respect, the importance of context and the modulation of pain by emotion is now widely recognised. In particular, stress, fear and anxiety exert potent, but complex, modulatory influences on pain (Asmundson *et al.*, 2009; Butler *et al.*, 2009; Ford *et al.*, 2008; Rhudy *et al.*, 2000; 2001; Wiech *et al.*, 2009). Generally, research suggests that positive emotions inhibit and negative emotions enhance pain (de Wied *et al.*, 2001; Dougher, 1979; Meagher *et al.*, 2001; Rhudy *et al.*, 2001; 2000; 2003a; 2003b). Investigations suggest that the interaction between emotion and degree of arousal can influence the impact on pain (Rhudy *et al.*, 2001, 2000; 2003a; 2003b). Positive emotions generally inhibit pain (regardless of arousal level), whereas, negative emotions with low-to-moderate arousal enhance pain and negative emotions with high arousal inhibit pain. Hence, differential levels of valence and arousal may determine whether an aversive event induces hyperalgesia or analgesia.

There is now a large body of evidence demonstrating that stress/fear induces a potent form of endogenous analgesia which is adaptive and evolutionarily preserved (for review see Butler *et al.*, 2009). Acute, highly arousing, intense stress or fear usually suppresses pain in rodents (Basbaum *et al.*, 1984a; Bodnar *et al.*, 1980; Bolles *et al.*, 1980; Fanselow, 1986; Lichtman *et al.*, 1990; Maier *et al.*, 1982; Watkins *et al.*, 1986) and in humans (Al Absi *et al.*, 1991; Flor *et al.*, 1999; Janal *et al.*, 1984; Janssen *et al.*, 1996; Malow, 1981; Pitman *et al.*, 1990; Rhudy *et*

*al.*, 2000; Willer *et al.*, 1981). Both conditioned (CS) and unconditioned (US) stressors have been shown to induce analgesia, phenomena referred to herein as fear-induced (or fear-conditioned) analgesia (FCA) and stress-induced analgesia (SIA), respectively. Exposing rodents directly to the US (such as brief foot shock) exhibits an active coping fight or flight response and analgesia on standard tests of pain reactivity and is known as SIA (Fanselow, 1984; Grau, 1984; Maier, 1989). Exposing a rat to a non-aversive stimulus or context that was previously paired with aversive stimulus (US) (such as foot shock) results in gross somatomotor defensive responses including hypoalgesia (FCA) (Finn *et al.*, 2004; Helmstetter *et al.*, 1993; Iwata *et al.*, 1988; LeDoux *et al.*, 1988a). Hence, FCA utilizes the principle of Pavlovian conditioning and is characterised by a robust decrease in nociceptive behaviour in rodent models which, at its peak, can suppress pain-related behaviour by as much as 90% (Finn *et al.*, 2004; Harris and Westbrook, 1995). SIA/FCA is thus a potent endogenous pain suppression response that occurs during or following exposure to an unconditioned or conditioned type of stressful or fearful stimuli. Exposure to such aversive environments is thought to result in recruitment and activation of the intrinsic descending inhibitory pain pathway discussed above that projects from the amygdala to the midbrain PAG and the brainstem RVM, terminating in the DHSC (for review see Vaughan, 2006).

However, as mentioned above, stress/anxiety does not invariably suppress pain; it can also induce increased sensitivity to nociception which is termed hyperalgesia. Evidence suggests that depending on the type of stress and pain model employed stress/anxiety can enhance pain in rodents (Bradesi *et al.*, 2005; Imbe *et al.*, 2006; Quintero *et al.*, 2003; Quintero *et al.*, 2000; Rivat *et al.*, 2007; Suarez-Roca *et al.*, 2006a; Vidal *et al.*, 1986) and in humans (Al Absi *et al.*, 1991; Cornwall *et al.*, 1988; Dougher, 1979; Rhudy *et al.*, 2006; Rhudy *et al.*, 2000; Schumacher *et al.*, 1984; Thompson *et al.*, 2008; Weisenberg *et al.*, 1984; Williams *et al.*, 2007). In general, more sustained but less intense stress in rodents (Imbe *et al.*, 2006) or moderately arousing state/trait anxiety in humans (Carter *et al.*, 2002; James *et al.*, 2002; Rhudy *et al.*, 2000) is often associated with enhanced pain, referred to herein as anxiety-related hyperalgesia (ARH) or stress-induced hyperalgesia (SIH).

Fear is an immediate alarm reaction to present/imminent/expected threat (i.e. there is certain expectation) characterized by impulses to escape, and results in a surge of sympathetic arousal (Barlow *et al.*, 1996) which mobilizes the organism to take action or assume defensive behavior (fight or flight response). In unconditioned or conditioned fear associated with exposure to a noxious/aversive stimulus, the rats typically exhibit intense fear-related behaviour (freezing and 22 kHz ultrasonic vocalization) and analgesia. The perceptual-defensive-recuperative model proposes that inhibition of pain under such conditions is vital because pain-related responses may compromise performance or interfere with other defensive responses, such as escape (Bolles *et al.*, 1980). This would appear to be the most appropriate adaptive response as there is a need to focus on other defensive behaviours to facilitate survival. In contrast, less intense stress results in anxiety which is a future-orientated emotion characterized by negative affect and apprehensive anticipation of potential threats (uncertain expectation) (Rhudy *et al.*, 2000). It has been suggested that the anticipation of pain activates brain regions in close proximity to brain regions activated by pain itself (Ploghaus *et al.*, 1999). This uncertainty results in hypervigilance, uneasiness and somatic tension leading to increased alertness and scrutiny towards the environment that enhances sensory receptivity contributing to hyperalgesia. In other words, in the absence of sufficient information as to the nature of threat or when the animal is not expecting an identifiable grave danger (e.g., being restrained) animals might have difficulty elaborating an orientated response. In this case, increased sensitivity to painful stimuli may appear more adaptive response than analgesia enabling the organism to detect any possible threat as early as possible. This is in agreement with the general adaptive model of injury-related behaviour (Walters, 1994) which suggests that when there is a high probability of injury, a fear state (active defensive response) is elicited that inhibits pain; in contrast, when the probability of injury is low, an anxiety state (a passive defensive response) and hyperalgesia result. The same principle was shown to apply in humans where anxiety/fear induced by an aversive event produces analgesia (Willer *et al.*, 1981; Beecher 1969; Rhudy *et al.*, 2003) but anxiety, occurring in the absence of knowledge regarding a forthcoming event is accompanied by hyperalgesia (Maier *et al.*, 1982; Rhudy *et al.*, 2006; Rhudy *et al.*, 2000).

### ***1.4.1 Animal models of emotional modulation of pain***

Numerous animal models have been designed to study various types of pain (acute, neuropathic, inflammatory, post operative, visceral and cancer-related) that are encountered in the clinic. There is also a plethora of pre-clinical models of stress, both acute and chronic. Animal studies investigating the interaction between stress/aversion/fear usually involve the combination of one or more stressors with one or more pain tests/models. While models of SIA/FCA can play a key role in dissecting out the exact mechanisms of potent endogenous analgesia and possibly identifying therapeutic targets for pain, models of ARH mimic the human chronic pain condition associated with chronic emotional disorders and thus prove useful in the search for mechanisms and novel targets of relevance to pain, anxiety and their co-morbidity.

#### ***1.4.1.1 Animal models of SIA***

As has been reviewed in detail by Butler and Finn (2009) numerous different aversive stimuli (which can be conditioned or unconditioned) have been used to model SIA/FCA (see **Table 1.1**). These include exposure to a mild aversive environment such as exposure to a novel arena, elevated plus maze, or aversive stimuli that may have a noxious component (such as footshock). Some aversive stimuli induce more psychological stress (eg. infantile isolation, social conflict) while others may be predominantly physical in nature (e.g. forced swimming, cold water immersion, or predators such as biting mice, biting insects, cats, or snake). These aversive stimuli are paired with noxious stimuli, such as chemical irritant formalin, radiant heat, tail pinches, and intracutaneous electrical current, constituting pain models which are used to assess level of nociception. In the case of models that employ conditioned stress in rodents (i.e. to model FCA), the unconditioned stressor (commonly footshock) is paired to the conditioned stimulus (such as a context, light or auditory cue) through Pavlovian conditioning.

#### ***1.4.1.2 Animal models of SIH***

A number of animal models have been devised to study SIH (See **Table 1.2**). Aversive stimuli or environments used to induce SIH include brief and mildly aversive but innocuous stimuli such as novelty (Vidal, 1982), vibration (Jorum,

1988) and holding (Vidal, 1982). Other aversive stimuli used are usually applied repeatedly over a prolonged period of time with the intention of inducing a mild form of stress and hypervigilance. The use of stressors like forced swimming as a model of ARH has a second advantage since immobility induced by inescapable swim stress is thought to be a model of human depression (Porsolt *et al.*, 1977) and patients with depression have increased pain sensitivity (Arnow *et al.*, 2006). Thus this model may have some validity as a model of depression and/or anxietyenhanced pain.

Visceral hyperalgesia is commonly observed in patients with conditions such as irritable bowel syndrome (IBS) and interstitial cystitis. Psychological stress is widely believed to play a major role in the precipitation or exacerbation of IBS (Delvaux, 1999). Response to colonic/bladder distention after a single or repeated stress was used to induce visceral hyperalgesia (Ait-Belgnaoui *et al.*, 2005; Bradesi *et al.*, 2002; Bradesi *et al.*, 2005; Gue *et al.*, 1997a; Robbins *et al.*, 2007; Toulouse *et al.*, 2000).

Because anxiety is clearly influenced by genetic factors, the possibility exists that in patients with ARH the symptoms may be related to a genetic predisposition to anxiety (i.e. trait anxiety as opposed to state anxiety). To investigate the role of trait anxiety on pain, some authors used different strains of rats known to have varying levels of baseline anxiety-related behaviour (e.g. Sprague Dawley; SD vs. Wistar Kyoto; WKY) and assessed their response to nociceptive stimuli (see section **1.4.5.1.2 below** for details).

#### **1.4.2 Human models of emotional modulation of pain**

Human research to understand the impact of anxiety/fear on pain perception commonly involved induction of anxiety or fear on healthy volunteers with subsequent pain testing. As in the case of animal models, stressors used to model SIA/FCA and ARH/SIH can overlap, albeit with some differences with respect to how they are applied. A detailed review of human models of SIA/FCA can be found in Butler and Finn (2009) and Table 1.2 below constitutes a detailed summary with references.

On the other hand, studies of ARH/SIH in humans focus on assessing pain responding in individuals with existing anxiety (trait) or experimentally induced anxiety. To study the attributional account of the relationship between anxiety and pain that relevant but not irrelevant anxiety intensifies pain, volunteers were exposed to laboratory induced general anxiety or pain-specific anxiety through instructions/warnings (Al Absi *et al.*, 1991; Benedetti *et al.*, 1997; Cornwall *et al.*, 1988; Dougher, 1979; Weisenberg *et al.*, 1984). Some authors, instead of inducing anxiety in the laboratory, classified healthy subjects from the population into high and low anxiety (trait anxiety) or high or low anxiety sensitivity (a fear of anxiety-related symptoms) using standard anxiety scales (such as Taylor Manifest Anxiety Scale) and assessed their response to painful stimuli (Thompson *et al.*, 2008). Subjects with high anxiety showed enhanced pain responding compared with those with low anxiety. A number of studies have examined the responses of patients with mood disorders to painful stimuli in the laboratory (see Table 1.2).

**Table 1.1 Summary of rodent and human models used to study SIA/FCA**

Stressors		painful stimuli	References
Rodents	Context associated with footshocks	Formalin, radiant heat	(Chance <i>et al.</i> , 1978, Hayes <i>et al.</i> , 1978, Helmstetter and Fanselow, 1987a,b)
	Social learning of fear to biting insects	Insect bites	(Kavaliers <i>et al.</i> , 2001)
	Footshocks	Formalin, radiant heat	(Akil <i>et al.</i> , 1976, Madden <i>et al.</i> , 1977), (Bodnar <i>et al.</i> , 1978)
	Continuous cold swim	Radiant heat	(Bodnar <i>et al.</i> , 1979)
	Intermittent cold swim	Radiant heat	(Fukuda <i>et al.</i> , 2001)
	Hemorrhagic shock	Formalin	(Costa <i>et al.</i> , 2005)
	Restraint stress	Nitroglycerin	(Lee and Rodgers, 1990)
	Elevated plus maze	Radiant heat	(Kavaliers, 1988)
	Exposure to predators	Radiant heat	(Puglisi-Allegra <i>et al.</i> , 1982)
	Social isolation	Radiant heat	(Rodgers and Randall, 1987)
	Defeat experience	Radiant heat	(Rodgers <i>et al.</i> , 1997)
	Predator odor	Radiant heat	(Kavaliers <i>et al.</i> , 2001)
	Social learning of fear to biting insects	Insect bites	(Rodgers <i>et al.</i> , 1983)
	Social conflict	Attack by intruders	(Wright <i>et al.</i> , 1985)
	Intraperitoneal injection of hypertonic saline	Radiant heat	(Wideman <i>et al.</i> , 1996)
	Food restriction	Radiant heat	(Flor <i>et al.</i> , 1999)
	Green light previously paired to mental arithmetic plus noise	Intracutaneous electric current	(Flor <i>et al.</i> , 2002)
Humans	Auditory stimulus previously paired to mental arithmetic plus noise	Intracutaneous electric current	(Janssen and Arntz, 1996)
	Spider phobics exposed to spiders	Intracutaneous electric current	(Rhudy and Meagher 2000)
	Re-exposure to handshocks	Radiant heat	(van der Kolk <i>et al.</i> , 1989)
	War veterans with post-traumatic stress disorder viewing combat video	Radiant heat	(Willer <i>et al.</i> , 1981)
	Re-exposure to footshocks	Flexion reflex	(Flor and Grusser, 1999)
	Exposure to mental arithmetic plus noise	Intracutaneous electric current	(Marchand <i>et al.</i> , 1991)
	Transcutaneous electrical nerve stimulation	Radiant heat	(Abdulhameed <i>et al.</i> , 1989)
	Peripheral electrical stimulation	Dental pain	(Walsh <i>et al.</i> , 1995)
	Transcutaneous electrical nerve stimulation	Tournequet ischemia	(Wyllie <i>et al.</i> , 1981)
	Footshocks	Flexion reflex	(Rhudy and Meagher, 2000)
	Handshock	Radiant heat	

Adapted (with modification) from Butler and Finn, 2009

**Table 1.2 Summary of rodent and human models used to study SIH/ARH**

	<b>stressor/source of anxiety</b>	<b>painful stimulus</b>	<b>References</b>
Rodents	Social threat	Formalin/radiant heat / acetic acid injection	(Andre <i>et al.</i> , 2005; Langford <i>et al.</i> , 2011)
	Water avoidance	Colorectal distension/thermal and mechanical stimuli	(Bradesi <i>et al.</i> , 2005; Robbins <i>et al.</i> , 2007; Schwetz <i>et al.</i> , 2004)
	Restraint stress	Rectal distension/TF/ hot tail immersion/TMJ formalin/HP	(Bardin <i>et al.</i> , 2009; Bradesi <i>et al.</i> , 2002; da Silva Torres <i>et al.</i> , 2003b; Dhir <i>et al.</i> , 2006; Gamaro <i>et al.</i> , 1998; Gameiro <i>et al.</i> , 2005; King <i>et al.</i> , 2007; King <i>et al.</i> , 2003)
	Holding/Novelty	Tail shock/mechanical stimuli	(Rivat <i>et al.</i> , 2007; Vidal <i>et al.</i> , 1982)
	Rotational stress	Formalin	(Boccalon <i>et al.</i> , 2006)
	Prenatal stress(restraint)	Formalin	(Butkevich <i>et al.</i> , 2006; Butkevich <i>et al.</i> , 2007; Butkevich <i>et al.</i> , 2001)
	Vibration	TF	(Devall <i>et al.</i> , 2009; Jorum, 1988)
	Foot or tail shock	Tail immersion/ colorectal distension/TF/ tail shock	(Geerse <i>et al.</i> , 2006; King <i>et al.</i> , 1999; Tyler <i>et al.</i> , 2007)
	Forced swim	Thermal stimuli, mechanical stimuli, formalin, HP, carrageenan	(Imbe <i>et al.</i> , 2010; Metz <i>et al.</i> ; Quintero <i>et al.</i> , 2003; Quintero <i>et al.</i> , 2000; Suarez-Roca <i>et al.</i> , 2008; Suarez-Roca <i>et al.</i> , 2006a)
	Noise stress	Bradykinin, adrenaline	(Khasar <i>et al.</i> , 2009; Khasar <i>et al.</i> , 2005)
	Exposure to cold	Mechanical stimuli /footshock/capsaicin/Freund's adjuvant	(Kawanishi <i>et al.</i> , 1997; Okano <i>et al.</i> , 1997; Omiya <i>et al.</i> , 2000; Satoh <i>et al.</i> , 1992)
	REM sleep deprivation	Mechanical stimuli	(Wei <i>et al.</i> , 2007)
Humans	Patients with mood disorders	Electrical pain/ thermal stimuli/ cold-pressor pain	(Adler <i>et al.</i> , 1993; Dworkin <i>et al.</i> , 1995; Otto <i>et al.</i> , 1989; Ward <i>et al.</i> , 1982)
	Preoperative state anxiety	Post operative analgesic consumption	(Johnston, 1980, Kain <i>et al.</i> , 2000; Scott <i>et al.</i> , 1983; Wise <i>et al.</i> , 1978)
	High and low trait anxiety	Thermal stimuli/mechanical stimuli	(Dougher, 1979; Thompson <i>et al.</i> , 2008)
	Footshock	Radiant heat/ lower level of shock	(Williams <i>et al.</i> , 2007; Bowers 1968)
	Threatening pictures	Immersing arms into hot water	(Rhudy <i>et al.</i> , 2006)
	Experimentally induced anxiety (pain relevant negative information)	Radiant heat/ cold pressor/foot shock /ischemic arm pain/mechanical pressure	(Rhudy <i>et al.</i> , 2000; Al Absi <i>et al.</i> , 1991; Benedetti <i>et al.</i> , 2006; Cornwall <i>et al.</i> , 1988; Dougher, 1979; Schumacher <i>et al.</i> , 1984; Weisenberg <i>et al.</i> , 1984)

TF-tail flick test; HP-hot plate test; TMJ-Temporomandibular joint

Moreover, patients that underwent elective surgical procedure were used to determine whether psychological variables such as preoperative anxiety can serve as predictors for postoperative pain responding (Ip *et al.*, 2009; Kain *et al.*, 2000; Martinez-Urrutia, 1975; Papaioannou *et al.*, 2009; Scott *et al.*, 1983; Vaughn *et al.*, 2007; Wise *et al.*, 1978).

It should be noted here that pain perception is variable across individuals and this could be a possible confounding factor when studying the interaction between anxiety and pain in humans. As ethical guidelines require that patients are aware they are in a “pain” experiment, experimental factors such as human attention and anticipation of a noxious stimulus can confound the results. Furthermore, though fear/anxiety is experimentally induced, the subjects are reassured that no real danger would happen to them unlike animal studies where subjects are almost certainly unaware of the nature and seriousness of the stimulus/threat they are about to experience.

#### ***1.4.3 Factors determining the outcome of stress on pain behaviour***

The modulatory influence of emotion on pain can be affected by a number of environmental variables or events. Significant evidence now suggests that whether a stressor induces analgesia or hyperalgesia depends on the type of stressor used and the method selected to measure the pain response. Some of these factors will be discussed in this section.

##### ***1.4.3.1 The repetitive nature of the stressor (chronicity vs. acuteness)***

A number of stressors that have been used to produce SIH are also used to produce SIA; one determining factor appears to be the repetitiveness of the stressor. Repetitive stress favors the induction of hyperalgesia in rodents. Hence, repeated exposure to loud sound (Khasar *et al.*, 2009), cold environment (Satoh *et al.*, 1992), restraint (Gameiro *et al.*, 2005), or swim stress (Quintero *et al.*, 2003) potentiates pain perception. In general, models that induce SIH involve chronic exposure to the stressor (days or weeks rather than minutes) implying that SIH may be the result of more chronic psychological stress rather than acute/physical stress as in the case of SIA. This observation is in line with

evidence from human studies showing strong co-morbidity between mood disorders and chronic pain (Asmundson *et al.*, 2009; Bair *et al.*, 2003). Studies have shown that rat models of chronic pain rather than acute pain are associated with elevated pain behaviours in the presence of an aversive stimulus (Rivat *et al.*, 2007). Children who experience the chronic stress of recurrent abdominal pain display stress-induced hyperalgesia to the cold pressor test (Dufton *et al.*, 2008). However, the effect of chronic vs. acute stress on pain perception is not consistent as acute stress can also produce hyperalgesia and prolonged stress may evoke analgesia. For example, the earliest studies by Vidal and colleagues showed that acute exposure to emotionally arousing non-noxious stress, such as inescapable holding, novel environments or vibration, was shown to produce hyperalgesia albeit in an immediate and transient fashion (Jorum, 1988; Vidal *et al.*, 1982; Vidal *et al.*, 1986); and exposure to chronic unpredictable stress were found to be analgesic (Pignatiello *et al.*, 1989; Pinto-Ribeiro *et al.*, 2004).

Paradigms which use chronic stress as opposed to acute stress, however, result in hyperalgesia that usually lasts longer than that induced by acute stress and seems to mimic the human chronic pain condition associated with chronic anxiety-related disorders. For example, repeated cold stress (Hata *et al.*, 1988; Kawanishi *et al.*, 1997; Okano *et al.*, 1997; Omiya *et al.*, 2000; Satoh *et al.*, 1992) was shown to facilitate the response to noxious stimuli for up to 3 days after the last exposure to cold stress (Satoh *et al.*, 1992). Other well characterized forms of chronic stress paradigms used to model SIH include chronic restraint stress (da Silva Torres *et al.*, 2003a; da Silva Torres *et al.*, 2003b; Imbe *et al.*, 2004) and repeated forced swim stress (Quintero *et al.*, 2000; 2003) which induced long-lasting hyperalgesia, the hyperalgesia at times lasting 28 days after the cessation of the chronic stress (Quintero *et al.*, 2000).

#### **1.4.3.2 Severity and relevance of the stressor**

It has also been proposed that the severity of the stressor differentially modulates pain sensitivity, such that more severe stressors evoke SIA, whereas less severe stressors evoke SIH. Supporting this proposal, in a model of social threat, acute stress evoked by the potential for actual physical aggression produced analgesia, whereas limiting physical contact between animals

eliminated the acute/intense stress and perhaps instead triggered psychological stress/anxiety from the mere presence of an unfamiliar stranger male, thereby producing SIH (Langford *et al.*, 2011). In addition, thermal pain reactivity is inhibited in rats after exposure to very severe shock, whereas hyperalgesia is observed after low to moderate intensity shock. Maier and colleagues showed that rats subjected to inescapable noxious shocks exhibited long-term analgesia whereas rats receiving an identical number of shocks but which were able to escape did not show analgesia (Maier *et al.*, 1982). In humans, the use of mild shock (5-mA) which was brief and aversive, but only mildly painful resulted in hyperalgesia (Williams *et al.*, 2007). In contrast, higher intensity of shock (12-mA and 70mA) (Willer *et al.*, 1981; Williams *et al.*, 2007) resulted in analgesia. Studies also demonstrated suppression of pain perception following major accidents or battles (Beecher 1969) vs. enhanced pain perception amongst those with anxiety disorders (Gureje, 2008). Thus, exposure to mild stress repeatedly might create uncertainty about future threats. Increased alertness and early detection of threats, including nociceptive stimuli, would then seem an appropriate adaptive response.

The notion that differential levels of severity may determine whether an aversive stimulus induces hyperalgesia or analgesia is consistent with theories of attentional influences on pain modulation by anxiety/fear (Arntz *et al.*, 1991, Arntz *et al.*, 1994; Janssen *et al.*, 1996), which suggest that stimuli that are severe enough to take the attention away from the pain suppresses pain and less intense stimuli increase the attention to the nociceptive stimulus enhancing pain perception. Whereas hyperalgesia may occur when the anticipatory anxiety is directed towards the pain itself (Benedetti *et al.*, 1997; Keltner *et al.*, 2006; Koyama *et al.*, 2005; Sawamoto *et al.*, 2000), analgesia may occur when anxiety is directed towards a stressor that shifts the attention from the pain (Terman *et al.*, 1986; Willer *et al.*, 1980). However, this is not always the case as fear/anxiety irrelevant to pain can both enhance and suppress pain depending on the intensity (Rhudy *et al.*, 2000).

#### **1.4.3.3 Type of pain model employed**

Anxiety-related modulation of pain may be influenced by the type of nociceptive stimulus used to evoke pain responding. While anxiety demonstrated no association with pain intensity during cold stimulation, a significant hypersensitivity was found during heat stimulation in humans (Thompson *et al.*, 2008). Badesi *et al.* (2005) described a differential modulatory role of repeated psychological water avoidance stress on visceral vs. somatic nociception in rats. Chronic water avoidance stress resulted in a transient somatic antinociceptive response but sustained visceral hyperalgesia (Bradesi *et al.*, 2005). Similarly, restraint stress reduced the duration of lick/guard responses to thermal stimulation (analgesic effect) while also increasing sensitivity to thermal stimulation (hyperalgesic effect), as assessed by learned escape responses (King *et al.*, 2003). It is worth noting also that several studies where nociception was measured a few minutes after the last stress session have found that acute SIA is reduced following repeated forced swim in rodents but hyperalgesia does not develop (Blustein *et al.*, 1997; Vaccarino *et al.*, 1997). In contrast, Suarez-roca *et al.* (2006b) showed hyperalgesia when nociception was assessed at 24h following the last forced swim. This discrepancy could be due to the overlapping of acute and repeated stress, which may confound the interpretation of the results (Suarez-Roca *et al.*, 2006b). While results of pain testing at the end of the last session of series of stress tests may reflect the impact of both chronic and acute stress, testing 24h later only reflect the impact of chronic stress. Together, these studies suggest that the type of the pain model or noxious stimulus used, parameters used to assess pain-related behavior, as well as the time point selected for the test determine the impact of stress on pain and affect the potency of analgesic or hyperalgesic effect.

In summary, the types of stressor, its intensity and duration, as well as the type of the pain model/test used influence the direction of pain modulation by stress, and affect not only the potency of analgesic or hyperalgesic effect but possibly also the neuronal mechanisms responsible for them. The literature suggests that the stress-regulatory circuit activated by a particular stressor is crucially dependent on stimulus attributes (Herman *et al.*, 1997).

#### **1.4.4 Clinical significance of emotional modulation of pain**

##### **1.4.4.1 Clinical significance of SIA**

FCA/SIA are mediated by potent intrinsic analgesic mechanisms. The ability of humans to utilize this form of powerful endogenous analgesia has clinical potential. A thorough understanding of FCA/SIA means identifying the neurochemical and molecular mechanisms involved with the aim of then targeting/harnessing those mechanisms for therapeutic purposes in the development of potent analgesic treatment. For obvious reasons induction of SIA/FCA *per se* is not likely to be a useful or ethically valid means of treating pain clinically. However, if it were possible to engage the same potent analgesic mechanisms that underpin SIA/FCA, but avoid induction of stress/anxiety/fear, then this could be a very useful therapeutic approach. As Harris and Westbrook described, fear is sufficient, but not necessary for FCA (Harris *et al.*, 1994). Other evidence also exists supporting the notion that it is possible to isolate the aversive aspect from the analgesia (Helmstetter *et al.*, 1987) and *vice versa* (Kinscheck *et al.*, 1984; Roche *et al.*, 2007).

It should also be noted that activation of endogenous analgesic mechanisms can be achieved with stimuli that are not aversive in nature. Rightly so, such stimuli are termed distracting and the analgesia they induce is called distraction-induced analgesia, as the stimuli are considered distracting rather than aversive. In fact, SIA could be a form of distraction-induced analgesia, the stress being a distracter. In experiments employing painful electrical stimulation applied to the ankle of arachnophobes exposed to a spider cue, high anxiety was associated with reduced pain ratings but this effect was lost when differences in subjective attention were corrected suggesting the importance of attention in SIA/FCA (Janssen *et al.*, 1996). Attention towards and away from painful stimuli have been shown to have different effects on perception of pain. Thus, some forms of SIA may have distraction component away from noxious stimuli by the aversive stimuli. From a clinical point of view, understanding the mechanism involved distraction-induced analgesia is vital as it is devoid of an aversive component and thus has a direct therapeutic implication. Indeed, techniques utilizing this principle of analgesia are currently used clinically to treat pain. Examples of distracting stimuli used so far to induce analgesia include counting objects

(Zeltzer *et al.*, 1991), light centrifugal rotation (Hayes *et al.*, 1978), listening to music (Fowler-Kerry and Lander, 1987), virtual reality environment (Dahlquist *et al.*, 2010; Das *et al.*, 2005; Hoffman *et al.*, 2011; Hoffman *et al.*, 2001; Malloy *et al.*, 2010; Sato *et al.*, 2010; Schmitt *et al.*, 2011), viewing picture of romantic partner (Younger *et al.*, 2010) and affective pictures (de Tommaso *et al.*, 2009). In humans, the use of virtual reality environment resulted in marked reduction in subjective pain complaints during painful clinical procedures including dental procedures (Hoffman *et al.*, 2001) and dressing of paediatric burns (Das *et al.*, 2005; Hoffman *et al.*, 2001; Schmitt *et al.*, 2011). Brain regions thought to be involved and identified using imaging techniques in clinical studies of distraction-induced analgesia include prefrontal cortex (PFC), anterior cingulate cortex (ACC), amygdala, PAG and RVM (Buffington *et al.*, 2005; Petrovic *et al.*, 2002; Tracey *et al.*, 2002; Valet *et al.*, 2004). An important role for the monaminergic system in attentional control has been demonstrated in clinical studies (Scholes *et al.*, 2006). It has been demonstrated that distraction-induced analgesia to formalin is accompanied by significant reduction in serotonin and dopamine metabolites in the medial prefrontal cortex in rats (Ford *et al.*, 2008). However, there is a paucity of studies investigating neurochemistry of non-aversive distraction-induced analgesia.

#### **1.4.4.2 Clinical significance of SIH**

Over the past 20 years, there has been growing interest in the interaction between persistent pain and anxiety. Psychiatric pathologies such as anxiety disorders are often believed to influence the perception of pain. Indeed, exaggerated pain states associated with high levels of anxiety is a situation widely seen in medical practice and psychosomatic disorders (Suls *et al.*, 1989; van den Hout *et al.*, 2001). Clinical studies have shown that anxiety is associated with an increased frequency of chronic pain complaints (Asmundson *et al.*, 2009; Atkinson *et al.*, 1991; Dworkin *et al.*, 1995; Kain *et al.*, 2000; Lautenbacher *et al.*, 1999; Palermo *et al.*, 1996). Correlational studies also indicate that anxiety is related to increased pain reports in high trait anxious subjects (Dougher, 1979; Malow *et al.*, 1987). For example, recent research indicates that PTSD is frequently accompanied by acute pain episodes as well as chronic musculoskeletal pain (Asmundson, 2002; Otis et al 2003, Shipherd

2007). It is also true that a significant proportion of patients with chronic pain meet criteria for an anxiety disorder. Fishbain *et al.* (1986) found generalized anxiety disorder to be the most prevalent of the anxiety disorders in patients with chronic pain, seen in approximately 15% of chronic pain subjects. Including generalized anxiety disorder, adjustment disorder with anxious mood, OCD, PTSD and agoraphobia, the prevalence of clinical anxiety among the population with chronic pain may be as high as 60% (Fishbain *et al.*, 1986). The co-occurrence of anxiety disorders and chronic pain amplifies the negative effects of each alone, often complicating the treatment and resulting in poor outcome (Asmundson *et al.*, 2008).

In addition, clinically, stress has a major impact on pain perception. For example, stressful events worsen the symptoms perceived by patients suffering from chronic pain (Conrad *et al.*, 2007; Fishbain *et al.*, 2006; Zautra *et al.*, 2007; Zaza *et al.*, 2002). Stress amplifies nociception in irritable bowel syndrome, headaches and abdominal pain (Alfven *et al.*, 2008; Bennett *et al.*, 1998; Boey *et al.*, 2002). Painful medical syndromes such as chronic shoulder/neck pain syndrome (Nilsen *et al.*, 2007), the complex regional pain syndrome (Grande *et al.*, 2004) and fibromyalgia (FM) (Davis 2001; Van Houdenhove *et al.*, 2006) are all strongly associated with or precipitated by stress. Furthermore, some anxiolytic drugs, such as gabapentin, a GABA analogue and paroxetine, a selective serotonin reuptake inhibitor, are effective in relieving pain in animal models as well as clinical pain (Chouinard *et al.*, 1998; Matsuzawa-Yanagida *et al.*, 2007; Pande *et al.*, 1999; Portenoy, 2000), indicating the close relationship between pain and anxiety and suggesting a common pathogenesis. Further research is required to establish the specific value of these treatment options in the context of chronic pain that occurs across the various anxiety disorders.

While there is convincing evidence for anxiety-related hyperalgesia and the massive clinical burden of anxiety-pain co-morbidity, little is known regarding the neurochemical or molecular mechanisms underpinning this close relationship between pain and negative affect. Development of animal models for these disorders is vital to understand mechanisms, discover novel targets, and for preclinical evaluation of candidate drugs. Such work may lead to

improved management of the many individuals who experience co-occurring anxiety and chronic pain and, thereby, reduce suffering and improve quality of life. In addition, understanding the exact mechanisms of action of clinically active compounds in current use for the management of stress-related chronic pain disorders is also equally important.

#### ***1.4.5.1 Genetics and emotional modulation of pain***

##### ***1.4.5.1.1 Genetics and SIA***

Inheritance of SIA in mice, studied using a selective breeding technique which artificially selects genes to change trait-relevant gene frequencies in an experimental population, has helped to advance our understanding of the genetic basis of variability that exists in pain response. Randomly mated mice were tested for nociception following swim stress and postswim latencies on the hot plate were used to select progenitors of low (LA) and high (HA) analgesia lines (Panocka *et al.*, 1986). Significant divergence in SIA magnitude between HA and LA mice was achieved in the first generation of selection, arguing both for the high heritability and involvement of few gene loci in mediation of this trait (Sternberg *et al.*, 2001). Indeed, Mogil *et al.* identified a sex-specific locus on chromosome 8 that is significantly associated with non-opioid SIA in female mice but not in males (Mogil *et al.*, 1997). However, complete abolition of SIA required more than 21 generations of selective breeding (Mogil *et al.*, 1996) indicating that SIA is very well conserved.

Selective breeding for high and low swim stress-induced analgesia was associated with high and low levorphanol-induced analgesia, respectively (Lutfy *et al.*, 1996; Marek *et al.*, 1993). Such breeding also exerted a striking influence on the magnitude of the opioid-mediated type of footshock analgesia (Marek *et al.*, 1987) and on stimulation-produced analgesia (Marek *et al.*, 1989) but had no effect on non-opioid SIA (Marek *et al.*, 1987) suggesting a high degree of common genetic determination in opioid and swim SIA. The data also suggest that individual differences in analgesic responsiveness to opiate drugs could result from genetically determined individual differences in endogenous pain inhibitory mechanisms (Marek *et al.*, 1993).

#### **1.4.5.1.2 Genetics and SIH**

One way to examine the influence of genetic factors on anxiety related hyperalgesia in rodents is to assess pain behaviour across inbred rat strains. The use of inbred strains makes it easier to dissociate genetic and environmental components of a trait because the animals within each strain are theoretically identical in their genotype. Using strains of rats with genetic differences in anxiety traits, studies have shown that the threshold for nociceptive response also appeared to be subject to genetic variation (Gunter *et al.*, 2000). Fecho and colleagues demonstrated significant strain differences in pain sensitivity in two inbred strains, Lewis and Fischer, and an outbred SD strain, which elicit different responses in tests of fear or anxiety (Fecho *et al.*, 2005). At baseline, Fisher rats were the most sensitive to mechanical stimulation (the von Frey monofilament test) and the least sensitive to noxious heat pain (the Hargreaves radiant heat test). Following intraplantar administration of carrageenan, Lewis rats showed the least, and Fisher rats showed the highest, thermal hyperalgesia and mechanical allodynia/hyperalgesia (Fecho *et al.*, 2005). In another study, Lewis rats displayed higher levels of avoidance of different types of anxiogenic stimuli (Ramos *et al.*, 1997) and enhanced hyperalgesia in the formalin test, compared with spontaneously hypertensive rat counterparts (Ramos *et al.*, 2002). The authors suggested a possibility of differences in the gene *Tac1r* between these two strains, a gene that encodes for the substance P receptor neurokinin (NK)1 receptor , known to influence both pain and anxiety (Ramos *et al.*, 2002).

High-anxiety WKY rats display a heightened level of anxiety in response to behavioral tests of stress and anxiety such as the acoustic startle, open field and elevated plus maze tests compared with other strains including SD, Wistar, spontaneously hypertensive and Fisher rats (Burke *et al.*, 2010, Glowa and Hansen 1994; Gentsch *et al.*, 1987; Paré, 1992). These rats also exhibit neurochemical differences in response to stress and anxiety tests compared with SD and Lewis rats (Burke *et al.*, 2010; Pardon *et al.*, 2002). In addition, WKY rats exhibit an exaggerated visceromotor response to innocuous colorectal distention compared with low/moderate-anxiety Fisher and Sprague–Dawley rats (Gunter *et al.*, 2000). Using peripheral nerve injury model, it has been

shown that the presence of depression-like behavior in WKY rats was associated with exacerbated mechanical allodynia compared to Wistar rats (Zeng *et al.*, 2008). Moreover water avoidance stress resulted in augmented urinary bladder hyperalgesia in WKY rats (Robbins *et al.*, 2007). Chronic stress effects were only observed in high-anxiety rats suggesting a genetic component in SIH (Robbins *et al.*, 2007). Thus, rats with high anxiety state showed increased sensitivity to colonic (Gibney *et al.*, 2010; Greenwood-Van Meerveld *et al.*, 2005; Gunter *et al.*, 2000; O'Mahony *et al.*, 2010) or urinary bladder distention (Robbins *et al.*, 2007) and somatic (Burke *et al.*, 2010; Zeng *et al.*, 2008) noxious stimuli. The availability of multiple models is useful as even if a certain neurobiological mechanisms underlie the altered pain perception in one animal model, alternative mechanism may underlie this response in the other models.

To date work on genes associated with anxiety-pain co-morbidity is lacking. It has been reported that in patients with surgical pain, polymorphisms in three pre-specified pain-mood candidate genes ( catechol-O-methyl transferase, serotonin transporter and brain-derived neurotrophic factor) were not associated with late postoperative change in mood or with a pain-gene interaction on mood (Max *et al.*, 2006).

#### ***1.4.6 Age and emotional modulation of pain***

##### ***1.4.6.1 Age and SIA***

Developmental research on the ontogeny of endogenous analgesic function has revealed differential rates of maturation depending on the type of stressor and the neural or hormonal mechanisms they engage. Front-paw shock analgesia (opioid) has been shown to be functionally mature by 28 days of age whereas hind-paw shock analgesia (non-opioid) reaches maturity after two months of age in the rat. However, the opioid analgesic systems activated by cold-water immersion and food deprivation are active in 10-day and 6-day-old rats, respectively (Hamm *et al.*, 1988). In fact, analgesia induced by water immersion occurs in rats as young as 3 days old (Stolberg *et al.*, 1995). Research in animal models has shown an increase (Hamm *et al.*, 1986) Ghirardi *et al.*, 1994), a decline (Bodnar *et al.*, 1988; Kramer *et al.*, 1986; Hamm *et al.*, 1987a) or no change (Bodnar *et al.*, 1988; Hamm *et al.*, 1986) in SIA as a function of age. In

3-, 11-, and 23-month old rats, there was a progressive increase in the analgesia displayed after cold-water exposure as the age of the rats increased (Hamm *et al.*, 1986). Another study also observed an age-related increase in SIA (Ghirardi *et al.*, 1994), suggesting that aged animals display an enhanced analgesic response. However, using an array of age cohorts, Bodnar and colleagues illustrated that aging produces differential decrements in the analgesic responses induced by different stressors (i.e. different parameters of footshock [continuous vs. intermittent] and cold-water swims [continuous vs. intermittent]) (Bodnar *et al.*, 1988). Moreover decreases in the analgesic response to cold water swim stress as a function of age have also been observed (Kramer *et al.*, 1986). An age-related reduction in the degree of endogenous analgesia produced by hind-paw shock was revealed in a scopolamine-sensitive manner. The effectiveness of scopolamine blockade declined with age, implicating the cholinergic system in this age-related decline in SIA (Hamm *et al.*, 1987a). In addition, forced walking SIA using a formalin-induced paw licking test was age-dependent and was sensitive to an NMDA receptor antagonist (Onodera *et al.*, 2001). In contrast, some authors have also reported no age-related change in SIA responses. For example, food deprivation-induced analgesia (Bodnar *et al.*, 1988) and cold-water induced analgesia (tested in 10-day, 28-day, and 3-month-old rats) (Hamm *et al.*, 1987b) were not affected by aging.

In humans, older adults demonstrated facilitation rather than inhibition of thermal pain during concurrent noxious cold stimulation while younger adults demonstrated expected diffuse noxious inhibitory control (DNIC) effects suggesting age-associated reductions in a form of endogenous analgesia (Edwards *et al.*, 2003). Electrical and thermal nociceptive stressors can elicit an analgesic response in the cold pressor test that declined with age (Washington *et al.*, 2000). In addition, healthy older subjects failed to demonstrate conditioned pain modulation and showed facilitation indicating decreased endogenous pain inhibition in this age group (Riley *et al.*, 2010). Such findings of reduced pain-modulatory capacity in the elderly may partially explain age-related differences in the prevalence, severity, and impact of chronic pain (Gibson *et al.*, 2004). However, clinical studies also suggest a relative decrease in the frequency and intensity of pain symptoms associated with myocardial complaints, visceral

infections, musculoskeletal conditions, and postoperative and malignant pain problems in adults of advanced age, suggesting a differential response depending on the type and intensity of noxious stimulation (Gibson *et al.*, 2001). In summary, it would appear that the influence of age on SIA depends on the species, and/or the type of painful or stressful stimuli, which can mediate SIA through activation of different neurobiological systems.

#### ***1.4.6.2 Age and SIH***

According to a recent study, the degree of co-morbidity between pain and depression did not differ with age (Arnow *et al.*, 2006). Most clinical studies have shown that co-morbidity between mood disorders and pain can occur in age groups ranging from 21- 75 yrs (for review see Arnow *et al.*, 2006). The first evidence of the occurrence of SIH in a pediatric pain population was demonstrated by Dufton and colleaugues (Dufton *et al.*, 2008). Children with recurrent abdominal pain who underwent through stressful interview and serial subtraction task exhibited lower levels of pain tolerance to the cold pressor. More work is needed both in clinical and pre-clinical experiments to investigate changes in ARH/SIH as a function of age.

#### ***1.4.7 Effect of environment and experience on emotional modulation of pain***

##### ***1.4.7.1 Effect of environment and prior experience on SIA***

Previous experiences such as exposure to stressful environmental conditions which may or may not be painful can affect sensitivity to SIA. Researchers have shown that both pre-natal and post-natal exposure to different types of stimuli/stressors such as food deprivation, sleep deprivation, restraint, foot shock, exposure to ethanol, exposure to morphine or exposure to heat and maternal separation altered the expression of SIA (for review see Butler and Finn, 2009). For example, exposure of pregnant rats to heat and restraint stress resulted in reduced cold water swim-induced analgesia in the offspring in a sex dependent manner (Kinsley *et al.*, 1988). Nine-month old rats, which had been exposed to the cold-water swim test when younger, demonstrated an increased tolerance to SIA (Arjune *et al.*, 1989). SIA was also shown to be dependent on factors such as circadian rhythm. Warm (opioid dependent) and cold (non-opioid dependent) swim SIA displayed by mice was dependent on circadian

rhythm with maximum SIA observed at night (Kavaliers *et al.*, 1988). In addition, evidence demonstrates that parasitic and other subchronic infections can have a significant impact on the expression of opioid and non-opioid SIA (Kavaliers *et al.*, 1997b). Thus, previous experience affects SIA in different directions depending on the type of experience, the time of experience (pre- or post-natal), gender, the duration of exposure and the type of nociceptive and aversive test employed.

#### ***1.4.7.2 Effect of environment and prior experience on SIH***

A limited number of studies have looked at the impact of prior experience and environmental factors on SIH. Prior inflammatory pain or/and opioid experiences may favour the development of pain vulnerability after innocuous environmental stress. In contrast to naive rats, repeated stress induced hyperalgesia in the paw pressure vocalization test lasted several hours in pain- and opioid-experienced rats (Rivat 2007). Prior pain experience has also been shown to enhance escape responding of rats to noxious thermal stimulation (Vierck *et al.*, 2010).

#### ***1.4.8 The role of gender on emotional modulation of pain***

Both experimental and clinical studies underscore the existence of sex-dependent differences in the perception and responsiveness to nociceptive stimuli. The incidence of several chronic pain conditions is more common in women than in men (Greenspan *et al.*, 2007). Women are more likely to experience recurrent pain, have lower pain thresholds and tolerance and feel pain with higher intensity when compared to men (Taub *et al.*, 1995; Aloisi *et al.*, 2006; Berkley, 1997; Rhudy *et al.*, 2005; Unruh, 1996; Wiesenfeld-Hallin, 2005). Sex-related differences in nociceptive processing and responsiveness have also been documented in animal models of pain (Aloisi *et al.*, 1994; Beatty *et al.*, 1970) in a manner sensitive to gonadal manipulations (Romero, 1986).

##### ***1.4.8.1 The role of gender in SIA***

A number of preclinical studies suggest SIA is sensitive to gender differences, gonadectomy differences and steroid replacement differences (Bodnar *et al.*, 1988). The magnitudes of continuous cold water swim (CCWS) and intermittent cold water swim (ICWS)-induced analgesia were significantly lower in female rats as compared to male rats indicating that both opioid (ICWS) and non-opioid (CCWS) forms of SIA are sensitive to gender differences, and suggest that male gonadal hormones may enhance analgesic responsivity (Romero *et al.*, 1986). Indeed, testosterone reversed the deficits in CCWS and ICWS analgesia observed in both castrated and ovariectomized rats (Romero *et al.*, 1987). These data indicate that gonadal steroids play a major modulatory role in swim SIA.

Moreover, in mice, there exists a marked gender difference in the underlying neurochemical mediation of SIA, the development of which is influenced by early post-natal environment (Sternberg *et al.*, 1995). A role for glutamate and glutamate receptors in SIA in a sex-dependent manner has been reported. The competitive NMDA receptor antagonist, NPC 1262, attenuated analgesia in the hot-plate test in male mice exposed to a biting fly attack, but not female mice (Kavaliers *et al.*, 1997b). Kappa opioid receptor-dependent footshock-induced analgesia in the tail flick test was also shown to be gender dependent in mice (Menendez *et al.*, 1994). Naloxone significantly reduced endogenous analgesia induced by intermittent cold water stress as measured by the tail-flick test in castrated males, but not ovariectomized females reflecting further differences in the endogenous pain-inhibitory responses as a function of gender (Romero *et al.*, 1987).

#### **1.4.8.2 The role of gender in SIH**

Although basal pain thresholds are similar in male and female Wistar rats, sex differences in pain processing became evident in the presence of a mild non-noxious anxiogenic stress (Devall *et al.*, 2009). While male rats showed both hyperalgesia and analgesia, female rats exposed to vibration induced stress developed a robust SIH only in late dioestrus phase of the menstrual cycle (Devall *et al.*, 2009). The authors suggested hormone-linked plasticity of receptor expression in circuits involved in regulation of anxiety and pain

processing to be central to such effects. Ovariectomy prevented partial restraint stress-induced visceral hyper-responsiveness to rectal distension without affecting the nociceptive response to rectal distension in basal conditions (Bradesi *et al.*, 2002). 17 $\beta$ -estradiol- or 17 $\beta$ -estradiol-plus-progesterone-primed ovariectomised rats exhibit similar partial restraint stress-induced visceral hypersensitivity as that seen in intact controls, indicating the modulatory role of ovarian steroids on stress-induced visceral hypersensitivity and suggesting a major role of estrogens in this effect (Bradesi *et al.*, 2002). Enhanced pain behaviour in the formalin test in prenatally stressed rats (exposed to immobilization during the last weeks of pregnancy) was more pronounced in females (Butkevich *et al.*, 2009; Butkevich *et al.*, 2006; Butkevich *et al.*, 2007), revealing sexual dimorphism in the effects of prenatal stress on tonic pain with increased vulnerability of inhibitory processes to prenatal stress in females. Furthermore, maternal deprivation-induced visceral hyperalgesia has been shown to be gender-dependent with females being more sensitive than males (Rosztoczy *et al.*, 2003).

In humans, biological, psychological and socio-cultural factors contribute to sex related differences in pain experience and must all be considered (Fillingim, 2000). Studies suggest that men and women cope differently with stress (Wallbott *et al.*, 1991). It has been suggested that differences in emotional processing may contribute to gender differences in pain sensitivity (Rhudy *et al.*, 2005). Indeed, co-morbidity between pain conditions and psychiatric problems was higher among women (Bingefors *et al.*, 2004), though others did not find such a gender-related effect (Arnow *et al.*, 2006).

#### ***1.4.9 The neurobiology of emotional modulation of pain***

Stress/fear inhibits or enhances pain sensation by activating neural pathways in the brain that engage various neurochemicals. The underlying mechanisms are likely to be dependent on the recruitment of neural substrates that are important in the descending inhibitory/facilitatory pain pathways, including the amygdala, PAG, the RVM and the DHSC. Opioid and non-opioid forms of emotional modulation of pain have been described and will be discussed here.

##### ***1.4.9.1 Opioids and emotional modulation of pain***

Opioids have been the mainstay of pain treatment for thousands of years, and they remain so today. After the discovery of opioid receptors, stimulation-produced analgesia and its naloxone reversibility strongly pointed to the existence of endogenous opioids in the early 1970s (Akil *et al.*, 1972; Akil *et al.*, 1976). Opioids such as heroin and morphine exert their effects by mimicking naturally occurring substances, called endogenous opioid peptides or endorphins. The endogenous opioid system is comprised of three main opioid G-protein coupled receptor families,  $\delta$ ,  $\kappa$ , and  $\mu$  which are all G-protein coupled, and the endogenous ligands such as dynorphins, enkephalins, endorphins and endomorphins (Akil *et al.*, 1984). Among other things, the diverse functions of this system include modulation of pain and anxiety/fear (Millan, 2002; Millan, 2003).

#### **1.4.9.1.1 The role of opioids in SIA**

In general, two major types of endogenous pain inhibitory systems are commonly believed to exist: Opioid and non-opioid mediated. A large body of evidence suggests a role for the opioid system in SIA/FCA (Akil *et al.*, 1986; Amit *et al.*, 1986; Basbaum *et al.*, 1984a; Harris *et al.*, 1994). Factors determining opioid dependency of SIA/FCA include warm (opioid) vs. cold (non-opioid) swim stress (Kavaliers *et al.*, 1988), front paw (opioid) vs. hind paw (non-opioid) footshock (Watkins *et al.*, 1982), intermittent (opioid) vs. continuous (non-opioid) foot shock (Lewis *et al.*, 1980) and uncontrollable (opioids) (Janssen *et al.*, 1997) vs. controllable stressor (non-opioids) (Janssen *et al.*, 1999).

Pharmacologic studies have shown that systemic or intra-cerebral administration of opioid receptor antagonists attenuates SIA/FCA in rats (Akil *et al.*, 1976; Fanselow *et al.*, 1989; Hart *et al.*, 1983); whereas, enhancing endogenous opioid level using catabolic enzyme (enkephalinase) inhibitors enhanced SIA in rats, in a  $\mu$ -opioid receptor dependent manner (Chipkin *et al.*, 1982; Greenberg *et al.*, 1982). The use of transgenic mice revealed an important role of  $\beta$ -endorphin over met-enkephalin (Kieffer, 1999; Rubinstein *et al.*, 1996) and the necessity of  $\mu$ - or  $\delta$ -opioid receptors for the expression of opioid-mediated SIA (Contet *et al.*, 2005).

The opioidergic system at different levels in the CNS has been implicated in SIA/FCA. In the PAG, social conflict induced SIA was associated with decreased  $\beta$ -endorphin-like immunoreactivity (Kulling *et al.*, 1988). Blocking  $\mu$ -opioid receptors in the vIPAG with naltrexone attenuated both SIA (Wiedenmayer and Barr, 2000) and FCA (Helmstetter *et al.*, 1990). In contrast, an infusion of morphine into the nucleus accumbens, but not into the caudate-putamen or prefrontal cortex, impaired the expression of a FCA in rats on the hot-plate test in a dose-dependent manner and was reversed by systemic or intra-accumbal infusion of naloxone (Westbrook *et al.* 1992). Microinjection of morphine into the amygdala also impaired FCA in rats exposed to a hot-plate apparatus in a dose-dependent, naloxone-sensitive manner (Good and Westbrook, 1995). In addition, expression of FCA in the tail flick test after pairing auditory CS and footshock, was dependent on  $\mu$ -opioid receptors in the RVM, but not  $\delta$ - or  $\kappa$ -opioid receptors (Foo *et al.*, 1999). Further support for the involvement of  $\mu$ -opioid receptors in the RVM in FCA arises from studies intra-RVM pretreatment with antisense oligodeoxynucleotides directed against  $\mu$ -opioid receptor mRNA attenuated FCA (Foo *et al.*, 2000b). Moreover, the  $\kappa$ -opioid receptor agonist, U69593, injected directly into the RVM, completely blocked expression of FCA (Foo *et al.*, 2000a). These results suggest differential role of these receptors in FCA. A role for the opioid signalling in the hypothalamus and thalamus in SIA has also been demonstrated (Kurumaji *et al.*, 1987) and an opioid-mediated pituitary mechanism subserving conditioned analgesia has also been described (Gaiardi *et al.*, 1983). However, shock-induced analgesia was seen in both hypophysectomized and sham-operated rats and was sensitive to naloxone, implicating opioids of central nervous system rather than pituitary origin in its mediation (Lewis *et al.*, 1981).

Spinal  $\mu$ ,  $\kappa$  and  $\delta$  receptors have all been shown to mediate SIA/FCA (Menendez *et al.*, 1993; Watkins *et al.*, 1992; Wiertelak *et al.*, 1994). Indeed, combined blockade of all three classes of opioid receptor in the spinal cord antagonized all of the non-opioid forms of SIA, suggesting that all forms of SIA may in fact be dependent on opioid receptor activation at the level of the spinal cord (Watkins *et al.*, 1992).

In humans, analgesia to nociceptive flexion reflex following footshock stress was reversed by naloxone, providing evidence for involvement of endogenous opioids in SIA (Willer *et al.*, 1980; Willer *et al.*, 1981). Study of SIA in war veterans with PTSD demonstrated that re-exposure to a stimulus resembling the original traumatic event increased pain tolerance to a thermal stimulus, an effect attenuated by naloxone (Pitman *et al.*, 1990). In addition, in a classical conditioning paradigm combining auditory stimulus with mental arithmetic plus white noise, conditional analgesia was, at least partially, mediated by the endogenous opioid system (Flor *et al.*, 2002). It is believed that exposure to conditioned or unconditioned stressors increases levels of endogenous opioids in different brain regions and in the spinal cord (Curtis *et al.*, 2001), particularly at the level of PAG, RVM and DHSC, and that these in turn act on opioid receptors to produce analgesia.

#### ***1.4.9.1.2 The role of opioids in SIH***

It was reported that long-lasting delayed swim stress-induced thermal hyperalgesia was prevented by systemic administration of  $\mu$ -opioid receptor antagonists (Suarez-Roca *et al.*, 2006b) suggesting that endogenous opioid systems may also paradoxically play a role in SIH. Pre-stress treatment with low doses of the opioid receptor antagonist, naloxone, during exposure to swim stress prevented the development of SIH indicating that repeated activation of  $\mu$ -opioid receptors during forced swim stress might be required for the induction of hyperalgesia (Suarez-Roca *et al.*, 2006b). It has been shown that illness induced by lithium or lipopolysaccharides results in hyperalgesia in the rat formalin and tail-flick tests (Wiertelak *et al.*, 1994) which was blocked by the opioid receptor antagonist, naloxone (McNally *et al.*, 2000). The attenuation of both hyperalgesia and analgesia with naloxone might be explained by the role opioid receptors play in both descending facilitation and inhibition of pain.

In Long Evans rats, stress-induced increased sensitivity to thermal stimulation after restraint stress was attenuated and enhanced following morphine and naloxone systemic administration, respectively. The authors suggested that the

endogenous opioid system mediates effects of stress on these responses by enhancing stress-induced hyporeflexia and opposing stress-induced hyperalgesia (King *et al.*, 2007). In contrast to enhanced antinociception following administration of U-50488H (a  $\kappa$ -opioid receptor agonist) noted in repeated cold stressed mice, the antinociception induced by DAMGO ( $\mu$ -opioid receptor agonist) or morphine was reduced suggesting that repeated cold stressed mice were hyposensitive to supraspinal  $\mu$ -opioid receptor-mediated antinociception, whereas their antinociceptive activities through kappa-opioid receptor were increased (Omiya *et al.*, 2000). In addition, it was demonstrated that repeatedly restrained rats displayed decreased responses to morphine requiring an increased dose to show the classic analgesic effect on nociception on the temporomandibular joint formalin test (Gameiro *et al.*, 2005). Opioid-mediated novelty-induced analgesia on tail flick latencies (Siegfried *et al.*, 1987) was not seen in repeatedly restrained rats (Torres *et al.*, 2001) suggesting that repeated restraint stress induces an alteration in the nociceptive response perhaps as a result of altered levels or release of endogenous opioids in these animals. In line with this idea, previous studies showed that chronic restraint stress could modify the activity of the opioid systems (for review see Drolet *et al.*, 2001). For example, the density of opioid receptors in the repeatedly restrained rats decreased significantly in CNS structures such as the spinal cord, frontal cortex and hippocampus (Dantas *et al.*, 2005). Indeed, Omiya et al. (2000) showed that hypofunction of the supraspinal  $\mu$ -opioid receptor may explain the hyperalgesic effect of repeated cold stress in mice (Omiya *et al.*, 2000).

Endogenous opioids are released in the central nervous system in response to noxious or aversive stressors (Curtis *et al.*, 2001). Thus, frequent release of endogenous opioids as a consequence of repeated exposure to stressors could lead to overactivation and desensitization and/or downregulation of opioid receptors resulting in a tolerance to the analgesic effects of endogenous opioids, which might be implicated in the hyperalgesia, and reduced response to morphine observed after repeated swim stress. Though it is well known that exogenous opioids induce analgesia, hyperalgesia can also occur after analgesia following opioid administration in rats (Laulin *et al.*, 1998; Laulin *et al.*, 2002) and in humans (Angst *et al.*, 2003). Exposure to stress in rats after prior pain or

fentanyl experiences two weeks earlier resulted in hyperalgesia (Rivat *et al.*, 2007). Thus exposure to increased levels of endogenous or exogenous opioids could have either analgesic or hyperalgesic effects on subsequent pain responding depending on the frequency, duration of exposure and prior experience.

However, it should be noted that activity of opioid receptors is strongly coupled to modulation of the inhibitory amino acid neurotransmitter GABA and the excitatory amino acid neurotransmitter glutamate (for review see Christie *et al.*, 2000). For example, tolerance to the analgesic effects of opioids is associated with hyperalgesia (Mayer *et al.*, 1999) and increased activity of *N*-methyl-d-aspartate (NMDA) receptors (Trujillo *et al.*, 1991). Thus, alterations in opioid and NMDA receptor function could be implicated in the development and maintenance of SIH.

#### ***1.4.9.3 The HPA-axis and emotional modulation of pain***

The hypothalamic-pituitary-adrenal (HPA) axis is comprised of the hypothalamus, the pituitary gland and the adrenal glands and represents the major hormone system responsible for maintenance of homeostasis following exposure to stress (Herman *et al.*, 2003; Tsigos *et al.*, 2002). When the stress response is inadequate or excessive, illness such as mood disorders can develop (Chrousos *et al.*, 1992; de Kloet *et al.*, 2005).

##### ***1.4.9.3.1 The role of the HPA-axis in SIA***

In work by Bodnar and colleagues, sham rats exhibited profound analgesia following initial cold water swim stress, while hypophysectomized rats did not display any cold water swim stress-induced operant escape shifts and significantly less cold water swim stress-induced analgesia indicating the involvement of the pituitary gland in the mediation of cold water swim stress-induced analgesia (Bodnar *et al.*, 1979). Functional blockade of the hypophyseal-adrenocortical system, produced by systemic administration of hydrocortisone, dexamethasone or by implantation of dexamethasone above the paraventricular nucleus of the hypothalamus, resulted in reductions in SIA (Filaretov *et al.*, 1995; Filaretov *et al.*, 1996; Mousa *et al.*, 1983). In addition,

both hypophysectomy and dexamethasone administration blocked the analgesic consequences of inescapable shock (MacLennan *et al.*, 1982).

It has been suggested that selective breeding for high and low swim-induced analgesia modifies the extent of HPA axis involvement in the generation of SIA. Thus, dexamethasone reduced swim-induced analgesia in HA mice, but not in LA mice (Panocka *et al.*, 1987). The chronic pretreatment with metyrapone potentiated cold swim SIA, an effect which was reversed by naloxone, suggesting that the corticosteroid modulation (of the HPA axis) may have a role in regulating SIA, and interacts with opioid mediated pain-inhibiting systems (Mousa *et al.*, 1981). SIA which is blocked by dexamethasone and hypophysectomy, is also blocked by adrenalectomy and reinstated with corticosterone therapy (MacLennan *et al.*, 1982). Moreover, adrenalectomy prevented hemorrhagic shock-induced analgesia, which was reversed by corticosterone administration (Fukuda *et al.*, 2007). However, FCA was not accompanied by changes in plasma corticosterone levels in rats (Finn *et al.*, 2006).

#### **1.4.9.3.2 The role of HPA-axis in SIH**

Using a model of intermittent sound stress in rats, it was suggested that stress-induced enhancement of hyperalgesia requires activity in both neuroendocrine stress axes: the sympathoadrenal (via release of adrenaline) and HPA axis (via release of corticosterone) (Khasar *et al.*, 2008; Khasar *et al.*, 2009). Hypophysectomy potentiated inescapable holding-induced hyperalgesia, but attenuated novelty-induced hyperalgesia (Vidal *et al.*, 1982). However, dexamethasone, which is known to block the stress-induced release of adrenocortico trophin hormone (ACTH) and endorphin from the anterior lobe of the pituitary did not affect novelty-induced hyperalgesia but enhanced hyperalgesia induced by holding (Vidal *et al.*, 1982). Thus, hypophyseal factors, not affected by dexamethasone and originating from the pituitary, may participate in novelty-induced hyperalgesia whereas analgesic factors originating in the anterior pituitary (e.g. opioids) appear to counteract the holding-induced hyperalgesia.

Corticotrophin-releasing factor (CRF) is a hypothalamic peptide that stimulates the synthesis and release of ACTH and beta-endorphin from the pituitary (Bonfiglio et al., 2011) and interacts with CRF receptors, subtype1 (CRF<sub>1</sub> receptor) and/or subtype 2 (CRF<sub>2</sub> receptor) (Bale et al., 2004; Perrin et al., 1999). It acts both peripherally, as a hormone, and centrally on various brain regions that mediate the central response to stress (i.e., hypothalamus, amygdala, locus coeruleus, dorsal raphe nucleus, and hippocampus). CRF is reported to play an important role in stress-induced visceral hyperalgesia (Taché et al., 2004). Peripheral administration of the selective CRF<sub>1</sub> receptor antagonist, CP-154526, prior to the water avoidance stress prevented delayed stress-induced colonic hyperalgesia (Schwetz et al., 2004). In addition, systemic administration of the selective CRF<sub>1</sub> receptor antagonist, NBI 35965, abolished the stress-induced visceral hyperalgesia (Million et al., 2003). In rats, central (i.c.v.) administration of CRF mimics the effect of restraint stress in increasing the number of abdominal contractions to rectal distension (visceral hyperalgesia) (Gue et al., 1997b), and the hyperalgesic effect of such stress was blocked by i.c.v. administration of the CRF antagonist alpha-helical CRF9-41. It has also been reported that CRF<sub>1</sub> receptors are involved in stress-induced visceral hyperalgesia in a rat model of neonatal stress (Schwetz et al., 2005).

In humans, peripherally administered CRF decreased perception thresholds and increased intensity ratings in response to rectal distension in healthy volunteers (Lembo et al., 1996). A more recent study in humans has also shown that alpha-helical CRF significantly reduced the abdominal pain evoked by electrical stimulation in patients with Irritable Bowel Syndrome (Sagami et al., 2004).

#### **1.4.9.4 GABA and emotional modulation of pain**

GABAergic neurones constitute the major mode of inhibitory transmission throughout the CNS. The inhibitory neurotransmitter, GABA, acts on two distinct types of receptor, GABA<sub>A</sub> receptors, ligand-gated ion channels, and GABA<sub>B</sub> receptors, G-protein-coupled receptors (Bowery, 1993; Johnston, 1996). GABA activity at postsynaptic GABA<sub>A</sub> receptors mediates hyperpolarization by an increasing Cl<sup>-</sup> flux. The GABA<sub>A</sub> receptor-ionophore complex also contains modulatory receptor sites for the benzodiazepines and barbiturates (Olsen et al.,

1986). GABA<sub>B</sub> receptors are located pre- and postsynaptically, and exert their effects by inhibiting voltage-gated calcium channels (thus reducing transmitter release) and by opening potassium channels (thus reducing postsynaptic excitability) (Bowery *et al.*, 2006; Richards *et al.*, 1984; Bettler *et al.*, 2004). GABAergic neurotransmission plays a key role in supraspinal modulation of pain (Enna *et al.*, 2006; Millan, 2002; Rea *et al.*, 2007) and fear (Berlau *et al.*, 2006; Davis *et al.*, 2002; Pare *et al.*, 2004; Rea *et al.*, 2009b; Reimer *et al.*, 2008; Rodriguez Manzanares *et al.*, 2005).

#### **1.4.9.4.1 The role of GABA in SIA**

A number of studies have shown a role for GABAergic involvement in FCA (Harris *et al.*, 1994; Harris *et al.*, 1996; Helmstetter, 1993) and SIA (Tokuyama *et al.*, 1992; (Houston *et al.*, 1997; Killian *et al.*, 1995). The effect of GABA receptor agonists/antagonists on SIA is dependent on the dose of drug used, the type of stress and the receptor subtype targeted. Muscimol, a GABA<sub>A</sub> receptor agonist, suppressed psychological SIA at low dose but tended to potentiate it at high dose, potentiated swim SIA dose-dependently and did not affect footshock SIA (Tokuyama 1992). Both bicuculline, a GABA<sub>A</sub> receptor antagonist, and picrotoxin, a Cl<sup>-</sup> channel blocker, dose-dependently suppressed psychological and footshock SIA with little or no effect on swim SIA (Tokuyama 1992). Baclofen, a GABA<sub>B</sub> receptor agonist, had no influence on SIA, while, CGP 35348, a further GABA<sub>B</sub> receptor antagonist, dose-dependently blocked footshock-SIA without affecting either psychological or swim SIA (Tokuyama 1992). Thus, GABAergic signalling plays an important role in the production of SIA with different forms of stress engaging the two receptor subtypes differentially. In male mice, a 30s exposure to 2-propylthietane, the major component of weasel odor (the odor of a predator) elicited analgesia on the hot plate test which was partially sensitive to the GABA<sub>A</sub> antagonist, bicuculline (Kavaliers *et al.*, 1997a). The reduction in formalin-induced nociception following exposure to an aversively conditioned environment was sensitive to the benzodiazepine midazolam (Harris and Westbrook 1995; Fanselow *et al.*, 1988; Harris *et al.*, 1996), an effect reversed by the benzodiazepine binding site antagonist flumazenil (Harris *et al.*, 1996). Hypoalgesia on the tail flick test induced by transfer stress was also slightly reduced by diazepam (Jakoubek,

1984). However, unlike the formalin test (Fanselow *et al.*, 1988; Harris *et al.*, 1996), on tail flick test the benzodiazepine diazepam did not affect conditioned analgesia to footshock (Jakoubek, 1984).

Studies investigating neural substrates involved in GABAergic modulation of FCA have revealed some of the discrete brain regions involved. For example, intra-amygdala and intra-PAG administration of benzodiazepines prevented the expression of FCA in rats (Harris and Westbrook 1995; Helmstetter, 1993). A recent microdialysis study revealed that GABA levels in the basolateral amygdala, but not in the dorsolateral PAG, were significantly lower in fear-conditioned animals. However, no specific FCA-related alterations in GABA efflux were observed (Rea *et al.*, 2009a). It has been suggested that non-opioid SIA induced by cold water swim stress might be related to alterations in the rates of general ligand-receptor interactions including GABA<sub>A</sub>/benzodiazepine system in the cerebral cortex, hippocampus and cerebellum during stress (Amitani *et al.*, 2005). A role for GABA<sub>A</sub> receptors in the spinal cord was shown in cold-water swim SIA in mice as indicated by attenuation following intra-thecal administration of the antagonists, picrotoxin and biculline (Killian *et al.*, 1995). In human volunteers, the analgesic effects of a repetitive stress induced by anticipation of pain (noxious footshock) were attenuated by diazepam (Willer *et al.*, 1986). Thus, both enhancing and suppressing GABAergic transmission appear to result in either potentiation or attenuation of SIA/FCA depending primarily on the type of stress. It appears that following unconditioned stress GABAergic transmission largely enhances analgesia while following conditioned-stress it attenuates analgesia.

#### **1.4.9.4.2 The role of GABA in SIH**

Exposure to stress alters GABAergic transmission in the CNS which in turn regulates the stress response (Verkuyl *et al.*, 2004; Verkuyl *et al.*, 2005). Stress is also suggested to decreases the activity of the GABA<sub>A</sub> receptor complex (Biggio *et al.*, 1990), an effect mimicked by the *in vivo* administration of different inhibitors of GABAergic transmission and antagonized by anxiolytic benzodiazepines (Biggio *et al.*, 1990).

In forced swim-stressed rats, spinal GABA release was reduced in association with behavioral hyperalgesia, both of which were prevented by pre-stress treatment with diazepam, a positive modulator of GABA<sub>A</sub> receptors (Suarez-Roca *et al.*, 2008). The anti-hyperalgesic effect of diazepam was blocked by flumazenil, a selective antagonist of benzodiazepine binding sites, suggesting the involvement of GABA<sub>A</sub> receptors. In the same experiment, in forced swim stressed rats, pre-stress treatment with diazepam blocked pain-induced overexpression of c-Fos-protein in laminae I–VI of ipsilateral lumbar dorsal horn in a flumazenil sensitive manner. This finding indicates an involvement of spinal GABA receptors in SIH. In addition, it has been reported that diazepam abolishes the immediate and transient thermal and mechanical hyperalgesia observed after brief exposure to non-noxious stressors such as a novel environment (Vidal *et al.*, 1982) or 15min exposure to restraint/vibration stress (Jorum, 1988). However, diazepam did not affect inescapable holding-induced hyperalgesia (Jorum, 1988). Thus, as is the case with SIA, the role of GABA receptors on SIH depends on the types of stressors involved, which in turn is likely to impact on GABAergic tone in key neural substrates involved in stress-pain interactions.

#### ***1.4.9.5 Glutamate and emotional modulation of pain***

Glutamate, the major excitatory neurotransmitter in the mammalian CNS, fulfils a vitally important role in information transfer and synaptic plasticity within the CNS. Exposure to aversive stimuli is characterized by alterations in extracellular levels of glutamate (Karreman *et al.*, 1996; Timmerman *et al.*, 1999) and a perturbation of glutamatergic transmission is implicated in the affective symptoms of several psychiatric disorders, including anxious states (Millan, 2003; Moghaddam, 2002). Glutamate is also very important in the sensitization of dorsal horn neurons which underlies long-term, painful states (Lerma *et al.*, 2001; Martin *et al.*, 2001) and is involved in mechanisms of descending pain modulation (Vanegas, 2004; Watkins *et al.*, 1994).

##### ***1.4.9.5.1 The role of glutamate in SIA***

A role for glutamate in FCA (Lee *et al.*, 2001) and SIA (Kavaliers *et al.*, 1997a; Kavaliers *et al.*, 1998; Kavaliers *et al.*, 1997b; Onodera *et al.*, 2001) has been

demonstrated. A non-opioid form of SIA induced by forced walking combined with the formalin test was sensitive to the NMDA receptor antagonist, LY-235959, albeit in an age-dependent manner (Onodera *et al.*, 2001). In contrast to a 30 min exposure to biting flies, which elicited opioid-mediated analgesia, a 5 min exposure to biting flies elicited analgesia that was antagonised by the competitive NMDA antagonist, NPC 1262, though this effect was only seen in male mice indicating a glutamatergic SIA which is sex-specific (Kavaliers *et al.*, 1998). A 30 second exposure to odor of a predator, an ethologically relevant threatening stimulus, elicited analgesia that was blocked by NPC 1262 (Kavaliers *et al.*, 1997b). It was shown that NMDA receptors in the BLA participate in FCA as bilateral infusion of the NMDA receptor antagonist, d,l-2-amino-5-phosphonovaleric acid, into the rat BLA attenuated analgesia to tone and context which were previously paired with footshock (Lee *et al.*, 2001). It appears that antagonising the glutamatergic system at the NMDA receptor attenuates SIA/FCA which implies that acute fear or stress engages the glutamatergic system to enhance descending inhibitory pain pathway activation.

#### ***1.4.9.5.2 The role of glutamate in SIH***

Mechanical hypersensitivity induced by REM sleep disturbance in a rat model of nerve ligation-induced neuropathic pain and sham controls was attenuated by spinal administration of a metabotropic glutamate receptor 5 (mGluR5) antagonist or the NMDA receptor antagonist (Wei *et al.*, 2007). In neuropathic animals, the anti-hyperalgesic effect was most pronounced with the mGluR5 antagonist. This work suggests a role for glutamate receptors in SIH and that mechanical hypersensitivity following REM sleep disturbance and peripheral nerve injury share common spinal mechanisms involving mGluR5 (Wei *et al.*, 2007). Administration of the NMDA receptor antagonist, BN2572, prior to innocuous stress completely prevented SIH in rats with prior pain and fentanyl experiences suggesting that a sustained NMDA receptor blockade is required to counteract the activation of NMDA-dependent pronociceptive systems induced by innocuous stress in pain and prior opioid-experienced rats (Rivat *et al.*, 2007). In addition, the NMDA receptor antagonist, ketamine, at a dose that did not alter rat behaviour in the hot plate test in non-stressed rats, prevented and reversed

SIH suggesting the involvement of NMDA receptors in both the initiation and maintenance of this phenomenon (Suarez-Roca *et al.*, 2006b).

Several studies have found that stresses such as restraint and forced swimming induce glutamate release (Engelmann *et al.*, 2002; Fontella *et al.*, 2004); and in rats subjected to repeated cold stress, the release of glutamate evoked by capsaicin was markedly increased in the DHSC (Okano *et al.*, 1997). This could lead to compensatory changes in the NMDA receptor system perhaps interfering with the descending pain pathway. In addition, NMDA receptor antagonists also prevent tolerance to morphine analgesia induced by repeated social defeat stress (Belozertseva *et al.*, 1998) and hyperalgesia induced by repeated administration of an opioid agonist (Mao *et al.*, 1998).

#### **1.4.9.6 Monoamines and emotional modulation of pain**

The monoamines (noradrenaline, serotonin and dopamine) remain the most intensively-investigated transmitters implicated in regulation of mood and descending control of pain. The discovery of multiple classes of receptors with divergent roles in the mediation of descending inhibition and descending facilitation has contributed to our expanding knowledge of mechanisms of endogenous analgesia and facilitation of pain (Millan, 2002). Anxiogenic and other stressful stimuli activate monoaminergic projections that heavily innervate corticolimbic regions involved in integrating the response to anxiety and are accompanied by emotional and autonomic manifestations of fear behaviour (Millan, 2003).

##### **1.4.9.6.1 The role of monoamines in SIA**

**1.4.9.6.1.1 Serotonin:** Systemic administration of quipazine, an agonist at 5-HT<sub>2A</sub> and 5-HT<sub>3</sub> receptors, enhanced anti-nociception following stress and BC-105, an antagonist at 5-HT<sub>2A</sub> and 5HT<sub>2C</sub> receptors, blocked the increase in stress induced tail-flick latency (Snow *et al.*, 1982). Systemic administration of the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT, had biphasic effects on open arm confinement-induced antinociception, antagonising the response at the lowest dose and enhancing it at the highest dose (Nunes-de-Souza *et al.*, 2000). Front paw, but not hind paw, footshock SIA was significantly attenuated by depletion

of spinal serotonin or by combined depletion of spinal serotonin and noradrenaline indicating a differential role of the monoamines in different forms of stress (Watkins *et al.*, 1984). In addition, exposure to predator odor produced analgesia that was partially sensitive to the 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT (Kavaliers *et al.*, 1997b).

Despite the well established role of the amygdala in stress/fear and pain, bilateral injections of 8-OH-DPAT or NAN-190 (5HT<sub>1A</sub> receptor antagonist) into the amygdala did not alter open arm-confinement induced antinociception (Nunes-de-Souza *et al.*, 2000). Repeated footshock-induced analgesia assessed by latency to tail withdrawal from heat was associated with increased frontal cortex serotonin turnover (Rosecrans *et al.*, 1986) while FCA was accompanied by reduced tissue serotonin levels in the cerebellum (Roche *et al.*, 2007).

**1.4.9.6.1.2 Noradrenaline:** Clonidine, an alpha<sub>2</sub> adrenoceptor agonist potentiated cold water stress-induced analgesia in a synergistic or additive manner (Bodnar *et al.*, 1983). However, Snow and colleagues showed that systemically administered clonidine markedly decreased SIA and phenoxybenzamine, an alpha<sub>1</sub> adrenoceptor antagonist, increased the peak and duration of SIA (Snow *et al.*, 1982). The alpha<sub>2</sub> adrenoceptor antagonist, yohimbine, also potentiated cold water swim stress analgesia on the hot plate and tail-flick test (Bodnar *et al.*, 1983). Furthermore, acute, but not chronic, pretreatment with the tricyclic antidepressant desipramine potentiated cold water swim stress analgesia as measured by the hot plate test (Bodnar, 1985). However, FCA was not associated with changes in noradrenaline (Finn 2006; Roche 2007)

At the level of the spinal cord, a role for serotonin and noradrenaline has been demonstrated in front paw footshock SIA (Watkins *et al.*, 1984). The analgesia induced by acute exposure to cold water swim stress was shown to co-vary with levels of brain noradrenaline and is reduced by lesions of the locus coeruleus (Bodnar *et al.*, 1983). These data implicate a role for the noradrenergic receptor system in SIA/FCA though there are equivocal data as to whether enhanced or subdued noradrenergic activity favours SIA/FCA.

**1.4.9.6.1.3 Dopamine:** When dopaminergic tone was increased with systemic administration of apomorphine (agonist to dopamine D1 and dopamine D2 receptors), a stress-induced increase in tailflick latency was markedly attenuated, whereas, systemic blockade of D2 receptors with haloperidol enhanced SIA (Snow et al., 1982). However, hypoalgesia on the tail flick test induced by transfer stress and conditioned analgesia upon re-exposure to an aversive context were both blocked by chlorpromazine, antagonist of D2 receptor (Jakoubek, 1984). Repeated footshock-induced analgesia was associated with decreased hypothalamic dopamine turnover (Rosecrans et al., 1986). Tissue levels of 3,4-dihydroxyphenylacetic acid and the 3,4-dihydroxyphenylacetic acid:dopamine ratio were significantly increased in the PAG while levels of dopamine in the thalamus were decreased in rats expressing FCA (Finn et al., 2006). In addition, FCA was accompanied by increased homovanillic acid (HVA) : dopamine (DA) ratio in the cerebellum (Roche et al., 2007). Overall, both enhancing and inhibiting monoaminergic signaling seem to enhance or reduce SIA/FCA depending on the type of stress and pain models used.

#### **1.4.9.6.2 The role of monoamines in SIH**

**1.4.9.6.2.1 Serotonin:** It has been reported that a cold stress paradigm that decreased the levels of both serotonin and 5-hydroxyindoleacetic acid in different brain regions including hypothalamus, midbrain, thalamus, pons and medulla in rats (Hata et al., 1991), induced hyperalgesia in mice which was suppressed by the systemic administration of 5-hydroxytryptophan, a precursor of serotonin and by L-3,4-dihydroxyphenylalanine, a precursor of catecholamines (Ohara et al., 1991). Administration of fluoxetine, a selective serotonin reuptake inhibitor, attenuated chronic restraint stress induced hyperalgesia in the formalin test in rats (Gameiro et al., 2006). In addition, forced swim-induced hyperalgesia was completely blocked by acute pretreatment with tryptophan, a precursor of serotonin, and long-term pretreatment with clomipramine and fluoxetine, an effect not attributed to their analgesic properties (Quintero et al., 2000). Restraint SIH to thermal stimuli was associated with increased pErk immunoreactivity in neurons of the RVM, three-quarters of which are serotonergic in nature. In the same experiment, protein

levels of tryptophan hydroxylase were significantly increased in the RVM (Imbe *et al.*, 2004). These data suggest that the hyperalgesia after chronic/subchronic stress might be mediated by changes in the activity of the central serotonergic system. Moreover, WKY rats exhibited ARH which was inversely correlated with serotonin and 5-HIAA levels in the hypothalamus

The effect of swim stress on serotonin release in the brain is regionally-specific and bidirectional. Microdialysis studies in freely moving rats have shown increased serotonin release in several brain regions, especially in the raphe magnus (Adell *et al.*, 1997; Hellhammer *et al.*, 1983; Ikeda *et al.*, 1985), following short-lived forced swimming. On the other hand, prolonged forced swimming (e.g. 30 min) diminishes the efflux of serotonin in the amygdala and lateral septum (Kirby *et al.*, 1995). Moreover, swim stress produced decreases in extracellular serotonin levels in the forebrain targets of the dorsal raphe nucleus, a major serotonergic nucleus (Adell *et al.*, 1997). In cold-stressed rats, the levels of both serotonin and 5-HIAA decreased in the hypothalamus, thalamus, midbrain, pons and medulla (Hata *et al.*, 1988). Current treatments for painful conditions such as temporomandibular disorders that show increased stress, depression, anxiety and somatization (Gatchel *et al.*, 1996; Jones *et al.*, 1997) utilize drugs, such as fluoxetine, that increase the levels or activity of serotonin (Stokes *et al.* 1997). Thus, potentially changes in the activity of central serotonergic systems might explain, at least in part, the bidirectional changes in nociception (analgesia and hyperalgesia) seen after different stress conditions.

**1.4.9.6.2.2 Noradrenaline:** Cerebral release of noradrenaline during stress has been implicated as a factor in expression of anxiety (Tanaka *et al.*, 2000), which in turn enhances pain sensitivity in human subjects (Carter *et al.*, 2002; Rhudy *et al.*, 2003b). In addition, repeated stress is associated with increased noradrenaline turnover (Tsuda *et al.*, 1986). Clonidine, an alpha<sub>2</sub> agonist and inhibitor of the synaptic release of noradrenaline, was shown to block vibration-induced hyperalgesia (Jorum, 1988), indicating that enhanced noradrenergic system activity is involved in this form of SIH. However, pre-treatment with milnacipran, a dual serotonin/noradrenaline uptake inhibitor, reversed repeated forced swim stress-induced muscle hyperalgesia without modifying pre-stress

muscle nociception, suggesting that enhanced central noradrenaline and/or serotonin reverses this phenomenon (Suarez-Roca et al., 2006a). Furthermore, chronic restraint SIH to thermal stimuli applied to the tail was associated with significantly decreased pErk immunoreactivity in the rat locus coeruleus, a major nucleus of noradrenaline in the brain (Imbe *et al.*, 2004).

**1.4.9.6.2.3 Dopamine:** At the moment, direct experimental evidence implicating the role of dopamine in stress/anxiety related hyperalgesia is lacking. However, existing evidence suggests a possible role of dopamine in ARH. Exposure to acute stressors increases activation of dopaminergic neurons within the ventral tegmental area (VTA) (Bannon *et al.*, 1983; Kalivas *et al.*, 1987) and release of dopamine in a number of brain regions, including the nucleus accumbens (Bertolucci-D'Angio *et al.*, 1990; Wu *et al.*, 1999). Rats exposed to chronic unavoidable stress exhibit decreased dopamine efflux in the nucleus accumbens shell, which is associated with a reduced activity of dopaminergic neurons (Gambarana *et al.*, 1999). Indeed, there is a disruption of both serotonergic and dopaminergic function that occurs within the nucleus accumbens following chronic stress, the impact on dopamine outlasting that of serotonin (for review see Wood, 2004). Fibromyalgia, a clinical disorder characterised by disturbance of emotion and nociception, is thought to embody a hypo-dopaminergic state. A decrease in the concentration of dopamine metabolites (along with those of serotonin and norepinephrine) in patients with fibromyalgia has also been shown (Legangneux *et al.*, 2001; Russell *et al.*, 1992). Future work investigating the role of the dopaminergic system in SIH/ARH is needed to improve our understanding.

#### **1.4.9.2 Endocannabinoids and emotional modulation of pain**

While the neurotransmitter and neuroendocrine systems discussed above play key roles in emotional modulation of pain, the endocannabinoid system is the focus of the research presented in this dissertation. Moreover, the endocannabinoid system, as discussed earlier above, is capable of modulating the activity of each of the other systems reviewed above. CB<sub>1</sub> receptors, the predominant cannabinoid receptor subtype present in the brain (Herkenham *et al.*, 1991), are located throughout brain regions that are key components of the

descending inhibitory/facilitatory pain pathway and stress/fear/anxiety circuitry. The endocannabinoid system has recently emerged as an important modulator of many neural functions including the control of fear- and pain-related behaviour (Finn, 2010; Guindon *et al.*, 2009b; Moreira *et al.*, 2009b). Both painful stimuli (Walker *et al.*, 1999) and stress/fear (Hohmann *et al.*, 2005; Marsicano *et al.*, 2002) have been shown to increase levels of endocannabinoids in relevant brain regions. Taken together these findings suggest an overlap between neural substrates and pathways involved in cannabinoid-mediated modulation of pain and aversion (fear, anxiety) and implicate the endocannabinoid system as an important common denominator. Furthermore, cannabinoids activate the descending PAG–RVM pathway via GABA-mediated disinhibition (Szabo *et al.*, 2005).

#### **1.4.9.2.1 The role of the endocannabinoid system in SIA**

To date, studies have repeatedly shown a role for the endocannabinoid system in endogenous analgesia including SIA/FCA. Electrical stimulation-induced analgesia in the dlPAG was blocked by rimonabant and occurred concomitantly with the release of endogenous anandamide (Walker *et al.*, 1999). Evidence from pharmacological studies, employing both systemic and intra-cranial administration of drugs, supports a role for the endocannabinoids in SIA. Intraperitoneal administration of the CB<sub>1</sub> receptor antagonist rimonabant or its analogue AM251, but not a CB<sub>2</sub> receptor antagonist, abolished a non-opioid form of SIA in assessed with the rat tail flick test after foot shock as an unconditioned stimulus (Hohmann *et al.*, 2005). Enhancement of cannabinoid SIA was also observed following systemic injection of FAAH inhibitors (AA-5-HT and Palmitoyl trifluoromethylketone) (Suplita *et al.*, 2005). The use of transgenic mice lacking the CB<sub>1</sub> receptor further implicate a role for endocannabinoids in SIA (Valverde *et al.*, 2000). CB<sub>1</sub> knock-out mice did not exhibit antinociception following a forced swim and showed a decrease in the immobility induced by the previous exposure to electric footshock (Valverde *et al.*, 2000). Similarly, in a model that combines the formalin test of tonic persistent pain with contextual fear in rats, systemic administration of rimonabant attenuated the fear-induced suppression of formalin-evoked nociceptive behaviour, demonstrating the first evidence for a role of CB<sub>1</sub>

receptors in mediating FCA (Finn *et al.*, 2004). Subsequent work revealed enhanced cannabinoid-mediated FCA following systemic administration of the FAAH inhibitor, URB597 (Butler *et al.*, 2008, 2011a). It has been proposed that the endocannabinoid system acts in concert with the endogenous opioid system to mediate the expression of FCA in a similar manner to the functional association between the endogenous opioid and cannabinoid systems during pain modulation (Cichewicz *et al.*, 2003; Welch *et al.*, 1999). Moreover, studies conducted in mice have suggested interactions between the endocannabinoid system and the cholecystokinergic system (particularly CCK2 receptors) are important for expression of unconditioned SIA (Kurrikoff *et al.*, 2008).

A role for the endocannabinoid system in the PAG (Hohmann *et al.*, 2005; Suplita *et al.*, 2005), BLA (Connell *et al.*, 2006) and RVM (Suplita *et al.*, 2005) and spinal cord (Suplita *et al.*, 2006) in SIA has been demonstrated. Microinjection of rimonabant into the PAG (Hohmann *et al.*, 2005) and BLA (Connell *et al.*, 2006) but not central nucleus of amygdala (where CB<sub>1</sub> receptors are largely absent) prevented non-opioid SIA induced by continuous footshock. However, neither the FAAH inhibitor, URB597, nor the MAGL inhibitor, URB602, enhanced SIA when administered into the BLA, at doses that potentiated SIA following administration into the dorsolateral PAG (Hohmann *et al.*, 2005; Connell *et al.*, 2006). Moreover, blockade of CB<sub>1</sub> receptors in the RVM attenuated cannabinoid SIA; whereas, pharmacological inhibition of FAAH enhanced SIA in a CB<sub>1</sub>-dependent manner (Suplita *et al.*, 2005). The rapid increase of 2-AG/AEA in the midbrain PAG after stress indicates that endocannabinoid release might be responsible for SIA (Hohmann *et al.*, 2005). Unlike SIA, FCA was not prevented by intra-BLA rimonabant (Roche *et al.*, 2009; Roche *et al.*, 2007) suggesting a differential role of CB<sub>1</sub> receptors in the BLA in FCA and SIA. Perhaps endocannabinoid-mediated FCA may occur downstream of the BLA. No study to-date has investigated the role of CB<sub>1</sub> receptors in PAG and RVM in FCA.

#### ***1.4.9.2.2 The role of endocannabinoids in SIH***

Despite the well-established role of the endocannabinoid system in anxiety and pain, very few studies have investigated the role of endocannabinoids in SIH, with the only studies to-date focusing solely on visceral hyperalgesia. Intraperitoneal administration of the CB<sub>1</sub> receptor agonist arachidonyl-2-chloro ethylamine (ACEA) significantly diminished the enhanced visceromotor reflex to colorectal distention and also attenuated change in electromyogram response in rats stressed by partial restraint, whereas, the CB<sub>1</sub> receptor antagonist/inverse agonist (rimonabant) had the opposite effect (Shen *et al.*, 2010). In the same experiment, a stress-induced up-regulation of colon CB<sub>1</sub> receptors was demonstrated. In another study, visceral motor response increased significantly in water avoidance stressed rats, indicating hyperalgesia (Hong *et al.*, 2009). Treatment of water avoidance stressed rats with the cannabinoid receptor agonist, WIN 55,212-2, prevented the development of visceral hyperalgesia. In the same experiment, levels of anandamide in the dorsal root ganglia of stressed rats were increased; while CB<sub>1</sub> receptor expression was decreased (Hong *et al.*, 2009). These results suggest that endocannabinoid signalling through CB<sub>1</sub> may play an important role in stress-induced visceral hyperalgesia. More work is needed to understand the role of endocannabinoids in other forms of SIH/ARH.

**Table 1.3 Summary of neurotransmitter and endocrine systems involved in SIA**

neurotransmitter	Effect on SIA	Mediated by	References
<b>opioids</b>	enhanced	$\mu$	Chipkin <i>et al.</i> , 1982, Greenberg <i>et al.</i> , 1982, Contet <i>et al.</i> , 2005, Wiedenmayer and Barr, 2000, Helmstetter <i>et al.</i> , 1990 Foo <i>et al.</i> , 1999, Foo <i>et al.</i> , 2000b, Lewis <i>et al.</i> , 1981, Wiertelak <i>et al.</i> , 1994, Willer <i>et al.</i> , 1980, Willer <i>et al.</i> , 1981
		$\kappa$	Menendez <i>et al.</i> , 1993
	enhanced	$\delta$	Contet <i>et al.</i> , 2005, Wiertelak <i>et al.</i> , 1994
	reduced	$\mu$	Westbrook <i>et al.</i> , 1992, Good and Westbrook, 1995
<b>HPA</b>	reduced	$\kappa$	Foo <i>et al.</i> , 2000a
	enhanced	pituitary	Bodnar <i>et al.</i> , 1979
		hypothalamus	Filaretov <i>et al.</i> , 1995, Filaretov <i>et al.</i> , 1996, Mousa <i>et al.</i> , 1983, MacLennan <i>et al.</i> , 1982, Panocka <i>et al.</i> , 1987
<b>GABA</b>		adrenal	MacLennan <i>et al.</i> , 1982, Fukuda <i>et al.</i> , 2007
	enhanced	GABA <sub>A</sub>	Tokuyama, 1992, Kavaliers <i>et al.</i> , 1997a, Killian <i>et al.</i> , 1995
		GABA <sub>B</sub>	Tokuyama 1993
	reduced	GABA <sub>A</sub>	Harris and Westbrook, 1995, Fanselow <i>et al.</i> , 1988, Harris <i>et al.</i> , 1996, Jakoubek, 1984, Helmstetter, 1993, Willer <i>et al.</i> , 1986
<b>Glutamate</b>	enhanced	NMDA	Onodera <i>et al.</i> , 2001, Kavaliers <i>et al.</i> , 1998, Kavaliers <i>et al.</i> , 1997b, Lee <i>et al.</i> , 2001
<b>Serotonin</b>	enhanced	5-HT <sub>2A/2C/3</sub>	Snow <i>et al.</i> , 1982
		5-HT <sub>1A</sub>	Nunes-de-Souza <i>et al.</i> , 2000, Kavaliers <i>et al.</i> , 1997b
	reduced	5-HT <sub>1A</sub>	Watkins <i>et al.</i> , 1984
<b>Noradrenaline</b>	enhanced	alpha-2	Snow <i>et al.</i> , 1982
	reduced	alpha-2	Bodnar <i>et al.</i> , 1983
		alpha <sub>1</sub>	Snow <i>et al.</i> , 1982
<b>dopamine</b>	enhanced	-	Bodnar, 1985
		-	Watkins <i>et al.</i> , 1984
	reduced	D <sub>1&amp;2</sub>	Snow <i>et al.</i> , 1982
<b>Endocannabinoids</b>		D <sub>2</sub>	Snow <i>et al.</i> , 1982
	enhanced	D <sub>2</sub>	Jakoubek, 1984
	enhanced	CB <sub>1</sub>	Valverde <i>et al.</i> , 2000, Finn <i>et al.</i> , 2004, Butler <i>et al.</i> 2008, 2011, Hohmann <i>et al.</i> , 2005, Suplita <i>et al.</i> , 2005, 2006, Connell <i>et al.</i> , 2006

$\mu$  , mu opioid receptor ;  $\kappa$  , kappa opioid receptor ;  $\delta$  , delta opioid receptor ; GABA<sub>A/B</sub>, gamma Aminobutyric acid A/B receptors ; NMDA, N-Methyl-D-Aspartic acid receptor; 5-HT<sub>1A/2A/2C/3</sub>, serotonin 1A/2A/2C/3 receptors; D<sub>1&2</sub>, Dopamine receptors 1/2; CB<sub>1</sub> , Cannabinoid receptor 1

**Table 1.4 Summary of neurotransmitter and endocrine systems involved in SIH**

	<b>Effect on SIH</b>	<b>Mediated by</b>	<b>References</b>
<b>opioids</b>	enhanced	$\mu$	Suarez-Roca <i>et al.</i> , 2006b, McNally <i>et al.</i> , 2000
	reduced	$\mu$	King <i>et al.</i> , 2007, Omiya <i>et al.</i> , 2000
<b>HPA</b>	Enhanced	pituitary	Vidal <i>et al.</i> , 1982
		CRF1	Schwetz <i>et al.</i> , 2004, Million <i>et al.</i> , 2003, Schwetz <i>et al.</i> , 2005
		CRF1/CRF2	Gue <i>et al.</i> , 1997b, Lembo <i>et al.</i> , 1996, Sagami <i>et al.</i> , 2004
<b>GABA</b>	Reduced	pituitary	Vidal <i>et al.</i> , 1982
	reduced	$\text{GABA}_A$	Suarez-Roca <i>et al.</i> , 2008, Vidal <i>et al.</i> , 1982, Jorum, 1988
<b>Glutamate</b>	enhance	mGluR5	Wei <i>et al.</i> , 2007
		NMDA	Rivat <i>et al.</i> , 2007, Suarez-Roca <i>et al.</i> , 2006b
<b>Serotonin</b>	reduced	-	Hata <i>et al.</i> , 1991, Ohara <i>et al.</i> , 1991, Gameiro <i>et al.</i> , 2006, Quintero <i>et al.</i> , 2000
<b>Noradrenaline</b>	enhanced	$\alpha_2$	Jorum, 1988
	reduced	-	Suarez-Roca <i>et al.</i> , 2006a
<b>dopamine</b>	-	-	-
<b>Endocannabinoids</b>	reduced	$\text{CB}_1$	Shen <i>et al.</i> , 2010, Hong <i>et al.</i> , 2009

$\mu$  , mu opioid receptor; CRF1/2, cortisol releasing factor 1/2;  $\text{GABA}_A$ , gamma Aminobutyric acid A receptors; mGluR5, metabotropic glutamate receptor 5; NMDA, N-Methyl-D-Aspartic acid receptor ;  $\text{CB}_1$ , Cannabinoid receptor 1

### ***1.5 Overall objectives of the research presented in this dissertation***

The main objective of the work presented herein was to improve our understanding of the role of the endocannabinoid system in the interaction between stress and pain. The first 3 results chapters (Chapters 2-4) examined the role of the endocannabinoid system in key neural substrates implicated in FCA and also investigated molecular mechanisms underpinning endocannabinoid-mediated FCA. Chapters 5 and 6 aimed to investigate the role of the endocannabinoid system in ARH in relevant brain regions.

General aims of the present studies are:

1. To characterize alterations in the levels of endocannabinoids, NAEs and molecular correlates during expression of conditioned fear, formalin-evoked nociceptive responding or FCA in stress- and pain-related rat brain regions
2. To examine the role of the endocannabinoid system in the dlPAG in formalin-induced nociceptive behaviour, fear-related behaviour in the presence of nociceptive tone, and FCA in rats
3. To characterise a model of trait ARH using WKY versus SD rats, two strains with different baseline emotionality, and determine the role of the endocannabinoid system in ARH

The work in this PhD project tests the hypothesis that enhanced endocannabinoid signaling in brain regions such as BLA, PAG and RVM during the expression of conditioned fear mediates FCA and decreased endocannabinoid signaling in key brain regions such as BLA, PAG and RVM mediates ARH.

***Chapter 2: Characterisation of brain regional levels of endocannabinoids and N-acylethanamines during expression of conditioned fear, pain or fear-conditioned analgesia in rats***

### **2.1 Introduction**

FCA is the suppression of pain responding which occurs during or following exposure to conditioned stressors/fear. It is an important survival response and a potent form of endogenous analgesia. Elucidation of the neurochemical and molecular correlates/physiology of FCA may help to further our understanding of pain- and anxiety-related disorders and could uncover therapeutic targets for these disorders. The endocannabinoid system is among a number of neurotransmitter/neuromodulatory systems that are thought to mediate FCA. Understanding the response of the brain's cannabinoid system to noxious or/and aversive stimuli is fundamental to understanding the physiological/pathophysiological role the system plays in chronic pain and anxiety/fear disorders as well as FCA.

Evidence exists that endogenously released cannabinoids have a pain modulatory role. For example, it has been demonstrated that painful stimuli increase AEA release within pain modulatory brain structures such as the PAG (Walker *et al.*, 1999). Endocannabinoid/NAE levels are also increased in the dorsal root ganglion (Mitrirattanakul *et al.*, 2006), spinal and supraspinal areas (Petrosino *et al.*, 2007) and sensory neurons (Agarwal *et al.*, 2007) in various rodent models of neuropathic pain. However, decreases or no alteration in endocannabinoid levels in the spinal cord have also been observed in the CFA paw inflammation model in mice (Agarwal *et al.*, 2007). Further evidence for a role of the endocannabinoid system in the regulation of nociceptive responding comes from pharmacological studies. The cannabinoid CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, increases pain responding in inflammatory and neuropathic, as well as acute pain models (Herzberg *et al.*, 1997; Richardson *et al.*, 1998; Strangman *et al.*, 1998). In comparison, pharmacological or genetic inactivation of endocannabinoid degradative enzymes generally reduces nociceptive behaviour and inflammation (Cravatt *et al.*, 2001; Jhaveri *et al.*, 2006; Lichtman *et al.*, 2004b; Maione *et al.*, 2007).

However, some studies have been unable to demonstrate an ‘endogenous cannabinoid tone’ in models of pain (Beaulieu *et al.*, 2000; Fox *et al.*, 2001).

In addition to modulating nociceptive responding, the endocannabinoids play a modulatory role in emotional states such as anxiety and fear (Viveros *et al.*, 2005). Conditioned fear is accompanied by increased levels of endocannabinoids in the BLA which was proposed to mediate extinction of aversive memories (Marsicano *et al.*, 2002). Levels of endocannabinoids are also altered during chronic restraint stress in the hypothalamus (Patel *et al.*, 2004). Both increased and decreased levels of endocannabinoids in the amygdala were seen depending on the number of restraint (Rademacher *et al.*, 2008). In the PAG, acute footshock stress elicited a rapid formation of the two endocannabinoids, 2-AG and AEA (Hohmann *et al.*, 2005). Data from preclinical studies have also demonstrated that the contents of the endocannabinoids in limbic and hindbrain regions are altered by a variety of stressful stimuli (Gorzalka *et al.*, 2008). Though results to date are still controversial, several studies suggest an anxiolytic role for the endocannabinoids. For instance, anxiety is increased by either the genetic disruption or pharmacological blockade of the CB<sub>1</sub> receptor in rodents (Haller *et al.*, 2002; Navarro *et al.*, 1997; Patel *et al.*, 2006a; Urigüen *et al.*, 2004); however, reports of anxiolysis have also been reported with the cannabinoid antagonist rimonabant inducing anxiolysis (Griebel *et al.*, 2005; Haller *et al.*, 2002; Rodgers *et al.*, 2003). In addition, genetic and pharmacologic blockade of CB<sub>1</sub> receptor reduced some aspects of anxiety (Degroot *et al.*, 2004). Pharmacological blockade of intracellular AEA degradation, or blockade of AEA reuptake, produced anxiolytic effects on rats tested in different anxiety assays (Bortolato *et al.*, 2006; Gaetani *et al.*, 2003; Hill *et al.*, 2006; Kathuria *et al.*, 2003; Moreira *et al.*, 2008; Patel *et al.*, 2006a; Rubino *et al.*, 2008b). In addition, intra-cerebral injection of anandamide has been reported to produce anxiolytic effects (Moreira *et al.*, 2007).

A role for the CB<sub>1</sub> receptor and the endocannabinoids in the expression of FCA (Finn *et al.*, 2004, Butler *et al.*, 2008) and SIA (Hohmann *et al.*, 2005; Suplita

*et al.*, 2005) in rats has also been demonstrated. It has been suggested that endocannabinoids, levels of which are increased in the PAG, following exposure to stress, mediate SIA by engaging the descending inhibitory pathway that propagates to the dorsal horn of the spinal cord (Hohmann *et al.*, 2005). The presence of CB<sub>1</sub> receptors, the primary CNS receptors of the endocannabinoid system, and endocannabinoids themselves in CNS regions associated with pain and regions related to the modulation of fear and anxiety (Di Marzo *et al.*, 2000; Herkenham *et al.*, 1991; Mackie, 2005; Pacher *et al.*, 2006) indicates that key components of the endocannabinoid system are expressed in the appropriate neuroanatomical locations to regulate pain- and fear-related behaviours as well as their interaction, particularly FCA/SIA. To date, research has demonstrated that the endocannabinoid system in the cortex (Hoot *et al.*, 2010; Lin *et al.*, 2009; Martin *et al.*, 1999), hippocampus (Bradshaw *et al.*, 2006; Campos *et al.*, 2010; de Oliveira Alvares *et al.*, 2010), amygdala (Hasanein *et al.*, 2007; Manning *et al.*, 2003; Marsicano *et al.*, 2002; Martin *et al.*, 1999; Roche *et al.*, 2007), PAG (Finn *et al.*, 2003; Lisboa *et al.*, 2008; Moreira *et al.*, 2009a; Resstel *et al.*, 2008; Walker *et al.*, 1999; Wilson *et al.*, 2008) and RVM (Meng *et al.*, 2004) are involved in modulation of fear/stress and pain behaviours. Thus far, studies have also established that the endocannabinoids in the BLA, PAG and RVM mediate analgesia expressed following exposure to unconditioned stressful stimuli (SIA) (Connell *et al.*, 2006; Hohmann *et al.*, 2005; Suplita *et al.*, 2005). However, little is known about the alterations in levels of endocannabinoids and NAEs during conditioned psychological stress/fear, pain and FCA in these corticolimbic or brainstem regions. The studies presented in this chapter sought to address this paucity of information.

The extracellular signal-regulated kinase (Erk, including Erk1 and Erk2) is a member of the MAPK signalling cascade that transduces a broad range of extracellular stimuli into diverse intracellular responses by both transcriptional and non-transcriptional regulation (Johnson *et al.*, 2002; Widmann *et al.*, 1999). Among the wide range of functions of Erk, is its role in neuronal plasticity (Impey *et al.*, 1999). CB<sub>1</sub> receptor activation stimulates the MAPK signal transduction pathway via phosphorylation of Erk1/2 (Bouaboula *et al.*, 1995). A

role for MAPK in the PFC in conditioned fear has been reported (Hugues *et al.*, 2006). The expression of conditioned fear is also associated with increased phospho(p)Erk expression in the amygdala, an effect which was CB<sub>1</sub> receptor-dependent (Cannich *et al.*, 2004). In addition, Erk is selectively activated (i.e. phosphorylated) by conditions that may cause persistent changes in pain sensitivity, such as formalin-induced inflammatory pain (Karim *et al.*, 2001). MAPK activation in the spinal cord, hypothalamus, amygdala and PAG has been demonstrated following exposure to noxious stimuli (Carrasquillo *et al.*, 2008; Carrasquillo *et al.*, 2007; Choi *et al.*, 2006; Gioia *et al.*, 2005; Imbe *et al.*, 2004; Imbe, 2004 2005, 2008). As Erk and MAPK are altered in stress/anxiety and pain, it is not surprising that changes in pErk expression levels in key brain regions may mediate FCA, a topic recently investigated. Alterations in levels of pErk1/2 in the amygdala (Butler *et al.*, 2008) and PFC (Butler *et al.*, 2011) are associated with FCA. However, pErk1/2 expression in other brain regions such as the PAG, hippocampus, prefrontal cortex, and thalamus was unchanged following FCA (Butler et al 2008). Moreover, pharmacological enhancement and attenuation of FCA were associated with reduced expression of pErk1/2 in the amygdala, arguing against a causal role for Erk1/2 signaling in the amygdala during expression of FCA. Here, we investigated the hypothesis that endocannabinoid-mediated FCA may be associated with MAPK activation in key supraspinal components (BLA, PAG and RVM) at a time point where FCA was expressed maximally.

The main objective of the work described in this chapter was to comprehensively characterise alterations in the levels of endocannabinoids and NAEs during expression of conditioned fear, formalin-evoked nociceptive responding or FCA in stress- and pain-related rat brain regions where CB<sub>1</sub> receptors and their endogenous ligands have been identified. The work described also investigated changes in the expression of pErk1/2 in the BLA, PAG and RVM in association with conditioned fear, pain responding and FCA. The work tests the hypothesis that levels of endocannabinoids and related lipids and levels of expression of pErk1/2 increase during fear and nociception.

## **2.2 Methods**

### **2.2.1 Animals**

Experiments were carried out on adult male Lister-Hooded rats (225–250g on arrival; Charles River, Kent, UK) maintained at a constant temperature ( $21 \pm 2^{\circ}\text{C}$ ) under standard lighting conditions (12:12h light: dark, lights on from 07.00 to 19.00h). All experiments were carried out during the light phase between 08.00h and 17.00h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

### **2.2.2 Experimental procedure**

The experimental procedure was essentially as described previously (Butler *et al.*, 2008; Finn *et al.*, 2004; 2006; Ford *et al.*, 2011; Rea *et al.*, 2009a; 2011; Roche *et al.*, 2007; 2009). It consisted of two phases, conditioning and testing, occurring 24h apart. Subjects were randomly assigned to groups, and the sequence of testing was randomized in order to minimize any confounding effects of testing procedure. On the conditioning day, rats were placed in a Perspex fear conditioning / observation chamber (30 x 30 x 40cm), and after 15s received the first of 10 footshocks spaced 60s apart (0.4mA, 1s duration; LE85XCT Programmer and Scrambled Shock Generator, Linton Instrumentation, Norfolk, UK). Fifteen seconds after the last footshock, rats were returned to their home cage. Controls not receiving footshock were exposed to the chamber for an equivalent 9.5 min period.

The test phase commenced 23.5h later when the subjects received an intraplantar injection of 50 $\mu\text{L}$  formalin (2.5% in 0.9% saline) or 0.9% saline (control group) into the right hind-paw under brief isoflurane anaesthesia (3% in O<sub>2</sub>; 0.5L/min). Rats were returned to their home cage until 30min post-intraplantar injection, after which they were placed back in the Perspex observation chamber to which they had been exposed during the conditioning phase. This design resulted in four experimental groups: fear-conditioned +

saline (FC-Sal); fear-conditioned + formalin (FC-Form); non fear-conditioned + saline (NFC-Sal) and non fear-conditioned + formalin (NFC-Form). A bat detector (Batbox Duet, Batbox, Steyning, West Sussex, UK) was used to detect ultrasonic vocalization in the 22kHz range, and behaviours were recorded for 3min with the aid of a video camera located beneath the observation chamber. The 3min post-fear induction time-point was chosen based on the data from a number of experiments in our laboratory (Butler *et al.*, 2008, Roche *et al.*, 2007, 2010) demonstrating robust expression of FCA at this time-point and published work demonstrating fear-induced increases in brain endocannabinoid concentrations at this time-point (Marsicano *et al.*, 2002). Rats were decapitated at the end of the test trial and the brain removed rapidly within 5min, snap-frozen on dry ice and stored at -80°C for subsequent cryo-sectioning and collection of tissue for quantitation of endocannabinoids, NAEs, and expression of phosphorylated or total Erk1/2.

### **2.2.3 Behavioural analysis**

Behaviour was analysed using the Observer XT 7.0 software package (Noldus Technology, Wageningen, the Netherlands), which allowed for continuous event recording over the duration of the trial. A trained observer blind to the experimental conditions assessed behaviour including the duration of freezing (defined as the cessation of all visible movement except that necessary for respiration), duration of 22kHz ultrasound emission, and general behaviours (walking, rearing and grooming). Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring (CPS) technique described by (Watson *et al.*, 1997). According to this method, pain behaviours are categorized as time spent raising the formalin-injected paw above the floor without contact with any other surface (C1), and holding, licking, biting, shaking or flinching the injected paw (C2) to obtain a CPS [CPS = (C1 + 2(C2)) / (total duration of analysis period)]. Post-formalin oedema was assessed by measuring the hind-paw diameter before and after formalin injection using Vernier callipers.

#### **2.2.4 Cryo-sectioning and tissue dissection**

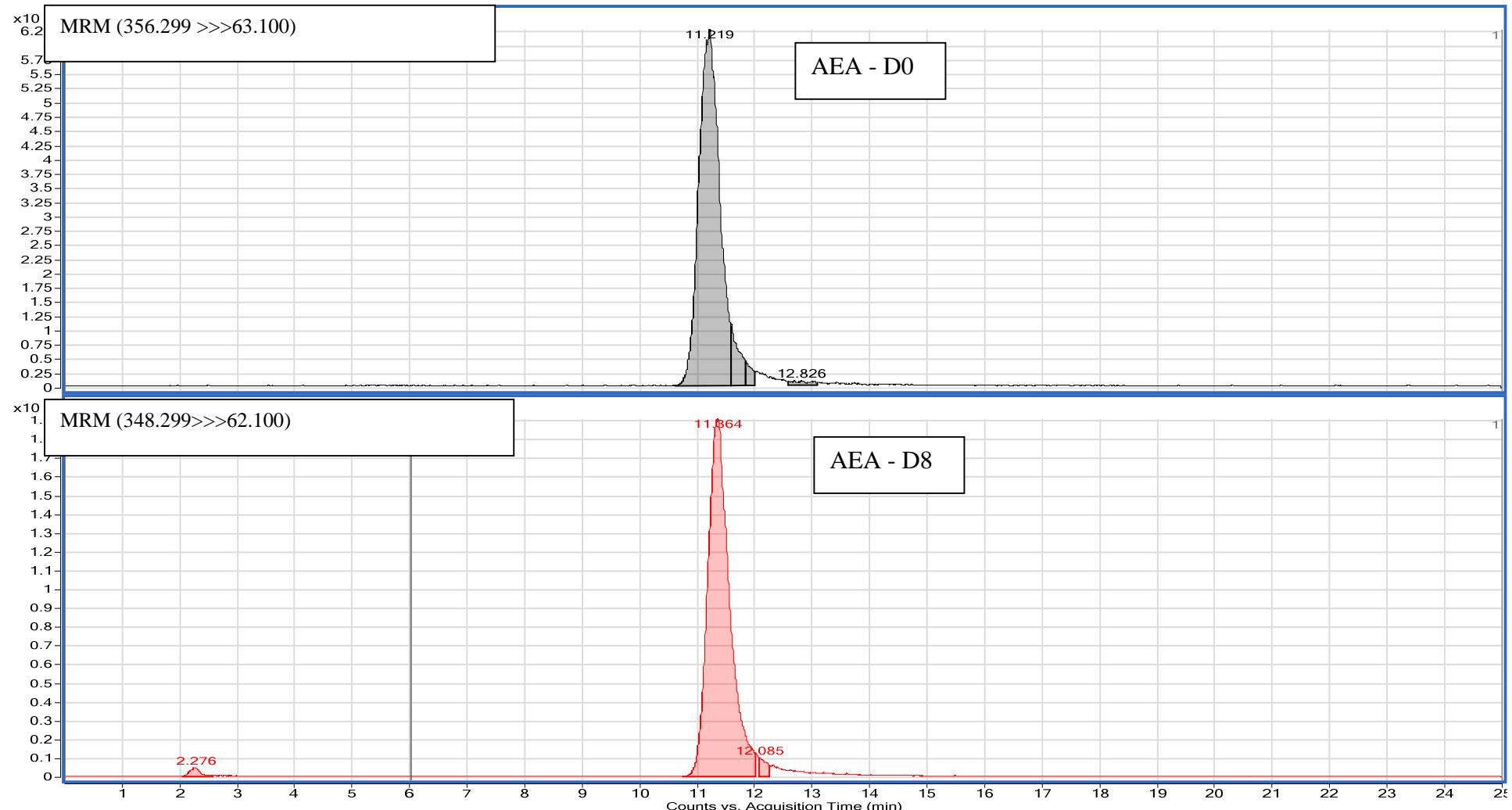
Frozen coronal brain sections (300 $\mu$ m) containing the PAG, BLA, hippocampus, prefrontal cortex, insular cortex and RVM were cut on a cryostat with reference to a rat brain atlas (Paxinos *et al.*, 1997). The following regions/nuclei were punched from the frozen sections using cylindrical brain punchers (Harvard Apparatus, internal diameter 0.75-2mm; 0.75mm for the different PAG regions and BLA, and 2mm for the rest): medial (m)PFC (Bregma, 3.7— -0.3mm), insular cortex (Bregma, 2.7— -0.7mm), dorsal(d)hippocampus (Bregma, -1.8— -8.0mm), ventral(v)hippocampus (Bregma, -7.3— -8.0mm), BLA (Bregma, -1.8— -3.3mm), dorsolateral(dl)PAG (Bregma, -5.8 — -8mm), ventrolateral(vl)PAG (Bregma, -7.3— -8.3mm), lateral(l)PAG (Bregma, -7.3— -8.3mm), and RVM (Bregma, -9.16 — -11.6mm). In order to understand effects of lateralisation, separate punches were taken for left and right for all regions mentioned above except for RVM and mPFC. Punched brain regions were weighed (range of weights of punched tissue: 4.5-20 mg depending on the region) and stored at -80 $^{\circ}$ C prior to extraction and determination of the concentrations of the endocannabinoids or NAEs by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) or determination of Erk expression using western immunoblotting.

#### **2.2.5 Quantitation of endocannabinoids and NAEs in discrete brain regions using liquid chromatography - tandem mass spectrometry (LC-MS/MS)**

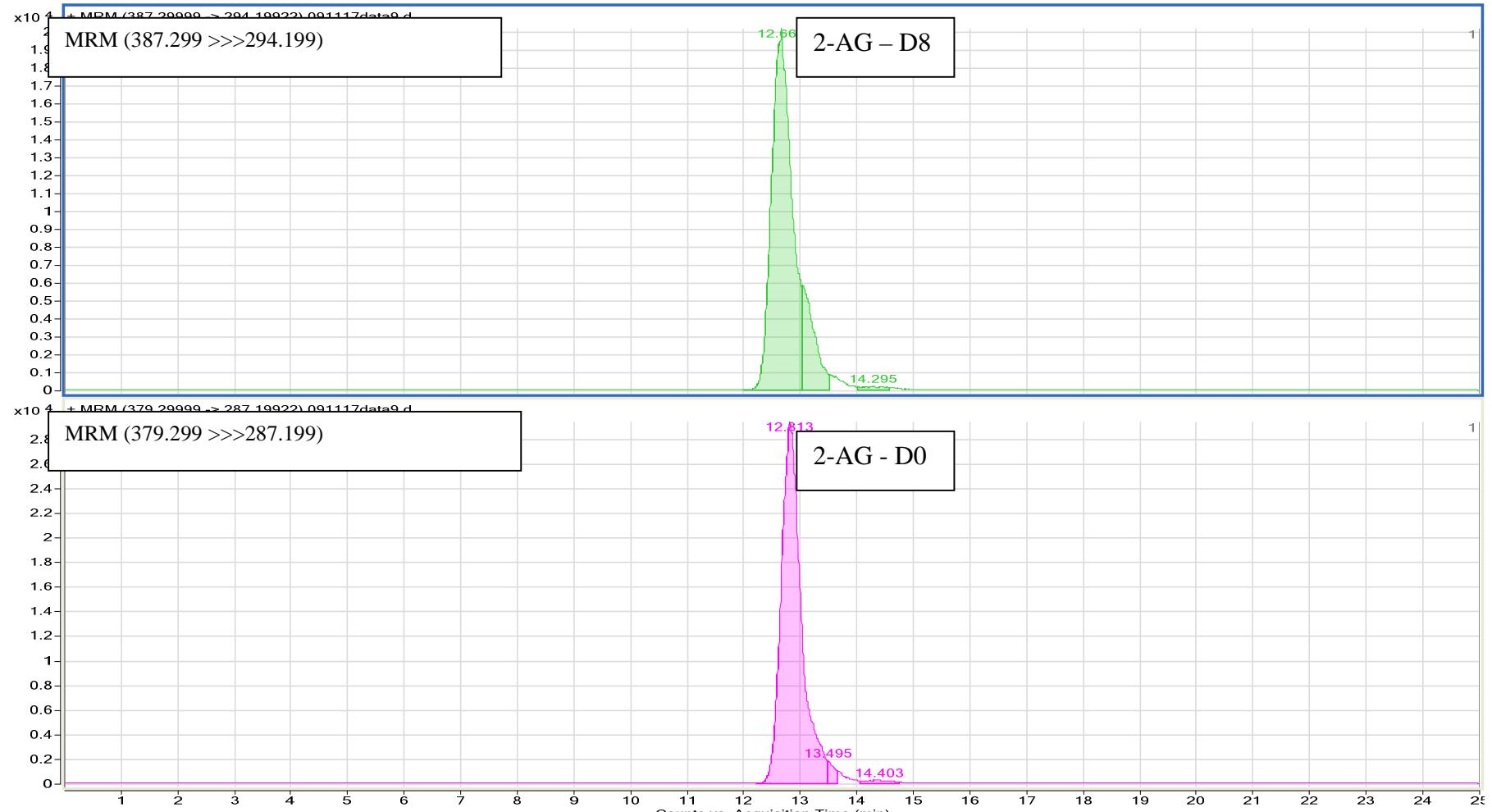
Tissue extraction was carried out using a lipid extraction method as follows. Each brain tissue sample was first homogenised for 3-5s in 400 $\mu$ L 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014nmol AEA-d8, 0.48nmol 2-AG-d8, 0.016nmol PEA-d4, 0.015nmol OEA-d2) using ultrasonic homogeniser/sonicator (Mason, Dublin, Ireland). Samples were kept on ice during the whole procedure. Homogenates were centrifuged at 14,000g for 15min at 4 $^{\circ}$ C and the supernatant was collected and evaporated to dryness after being spun for 90min in a centrifugal evaporator. Lyophilised samples were re-suspended in 40 $\mu$ l 65% acetonitrile and 2 $\mu$ l injected onto a Zorbax® C18 column (150 × 0.5mm internal diameter) from a cooled autosampler maintained at 4 $^{\circ}$ C (Agilent Technologies Ltd, Cork, Ireland).

Mobile phases consisted of A (high pressure liquid chromatography (HPLC) grade water with 0.1% formic acid) and B (acetonitrile), with a flow rate of 12 $\mu$ l/min. Reversed-phase gradient elution began initially at 65% B and over 10min was ramped linearly up to 100% B. At 10min, the gradient was held at 100% B up to 20min. At 20.1min, the gradient returned to initial conditions for a further 10min to re-equilibrate the column. The total run time was 30min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.36min, 12.8min, 14.48min and 15.21min respectively (see **Fig 1**). Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Instrument conditions, in particular source parameters such as fragmentor voltage and collision energy, were optimised for each analyte by infusing standards separately. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H<sup>+</sup>] of the analytes of interest and the deuterated form of the internal standard (MRM spectra and mass-to-charge (m/z) ratios of each analyte of interest and its corresponding internal standard are displayed in **Fig 2.1**). Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was calculated using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd, Cork, Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng per mg of tissue by dividing the weight of the punched tissue. To express values as nmol or pmols per mg the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmole or pg/pmol. Linearity (regression analysis determined R<sup>2</sup> values of 0.99 or greater for each analyte) was determined over a range of 18.75ng to 71.5fg except for 2-AG which was 187.5ng-715fg (see **Fig**

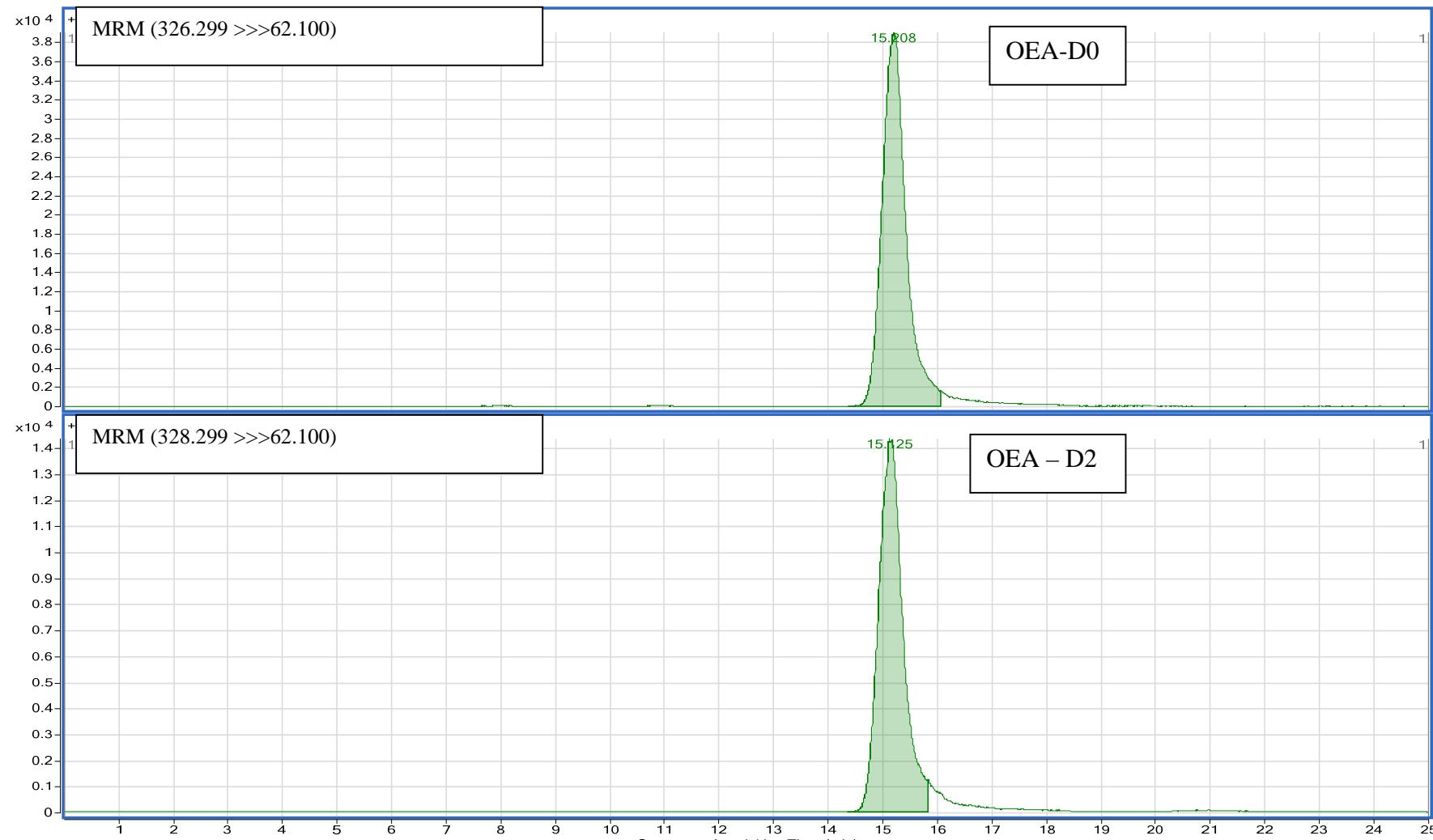
**2.2).** The limit of quantification was 1.32 pmol/g, 12.1 pmol/g, 1.5 pmol/g, 1.41 pmol/g for AEA, 2AG, PEA and OEA respectively.



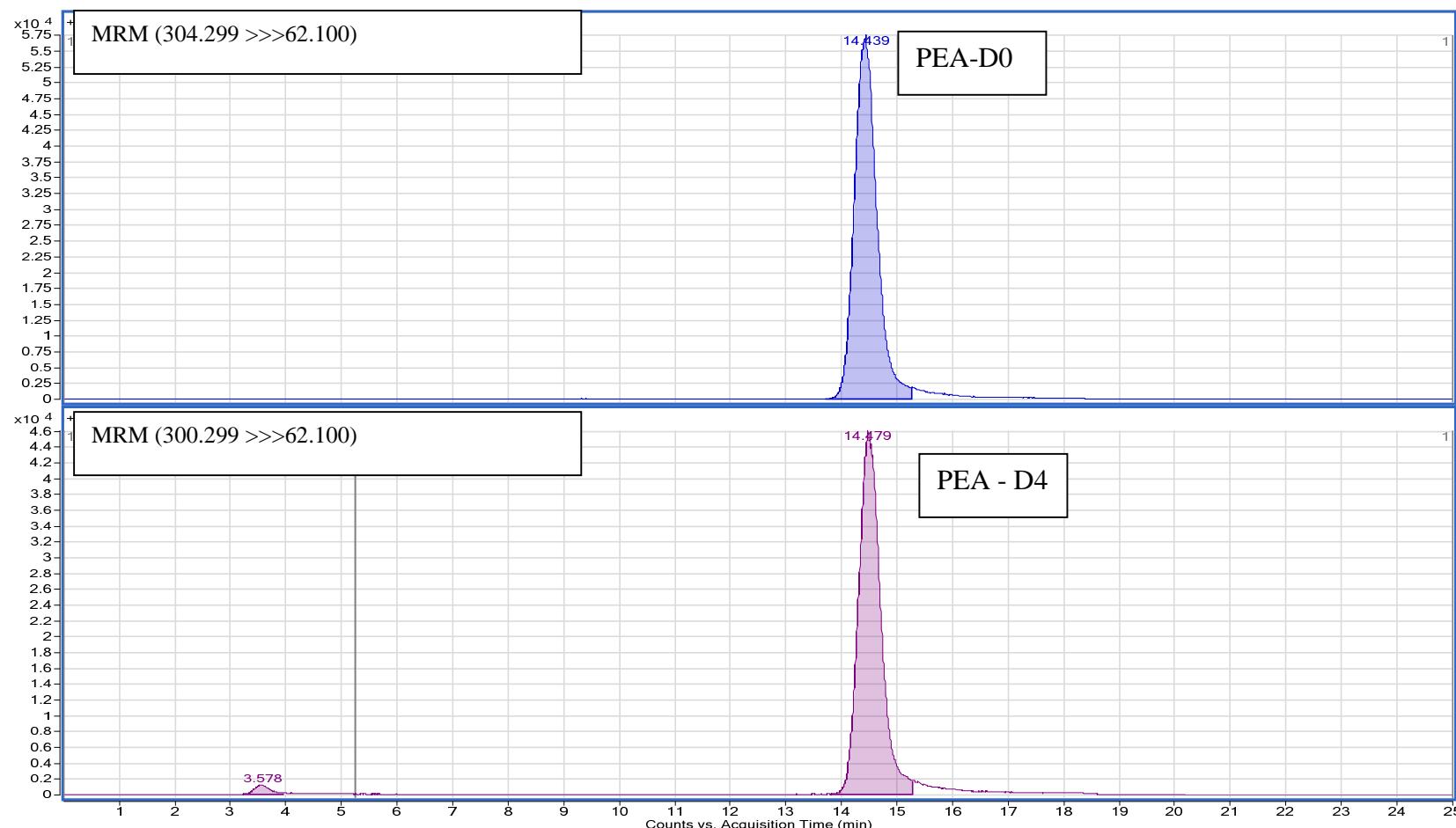
A)



B)



C)

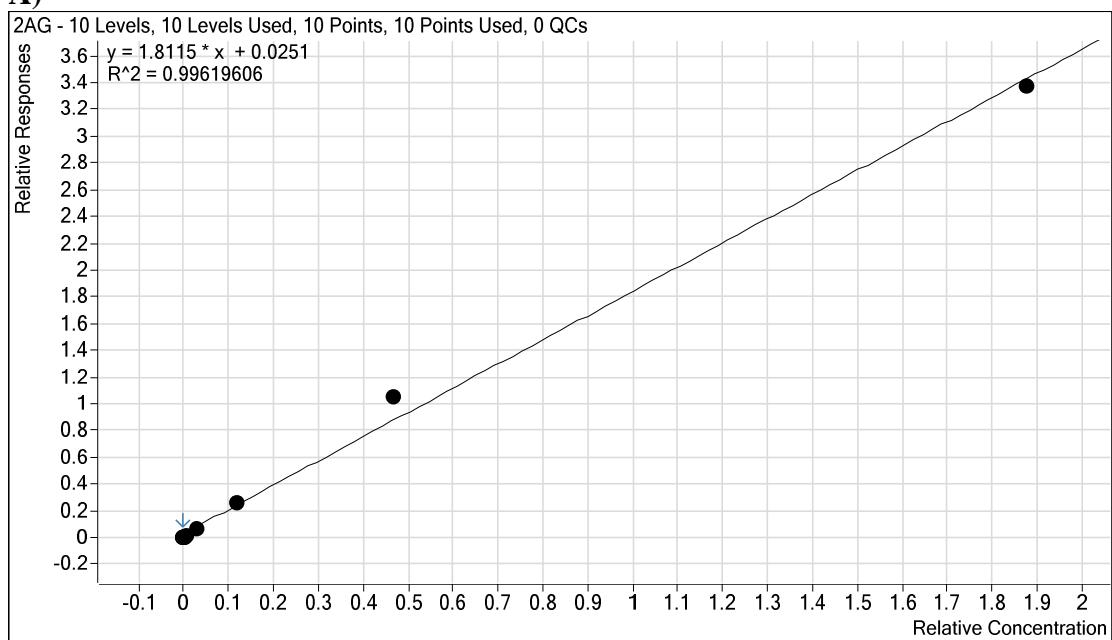


D)

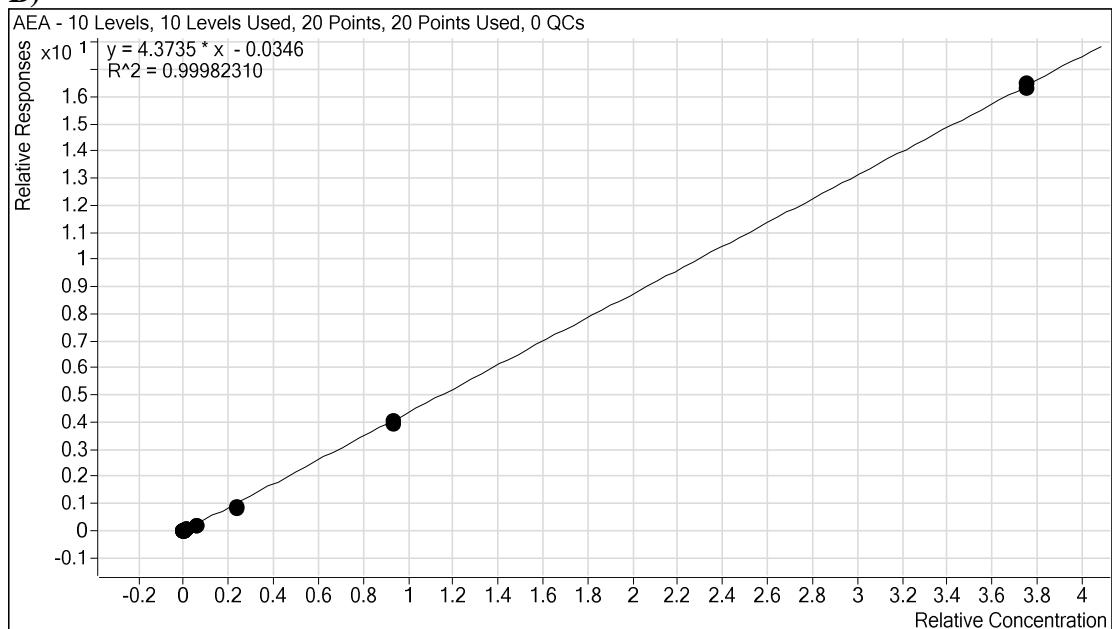
**Figure 2.1** MRM spectra and mass to charge ratios of each analyte of interest and its corresponding internal standard A) AEA, B) 2-AG, C) OEA and D) PEA

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**A)**



**B)**



**Figure 2.2** Sample 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard for A) 2-AG and B) AEA; Relative response on the y-axis is the ratio of peak area of undeuterated analyte to peak area of deuterated analyte; whereas, relative concentration on the x-axis is the ratio of amount in ng of undeuterated analyte to amount in ng of deuterated analyte.

### **2.2.6 Western immunoblotting**

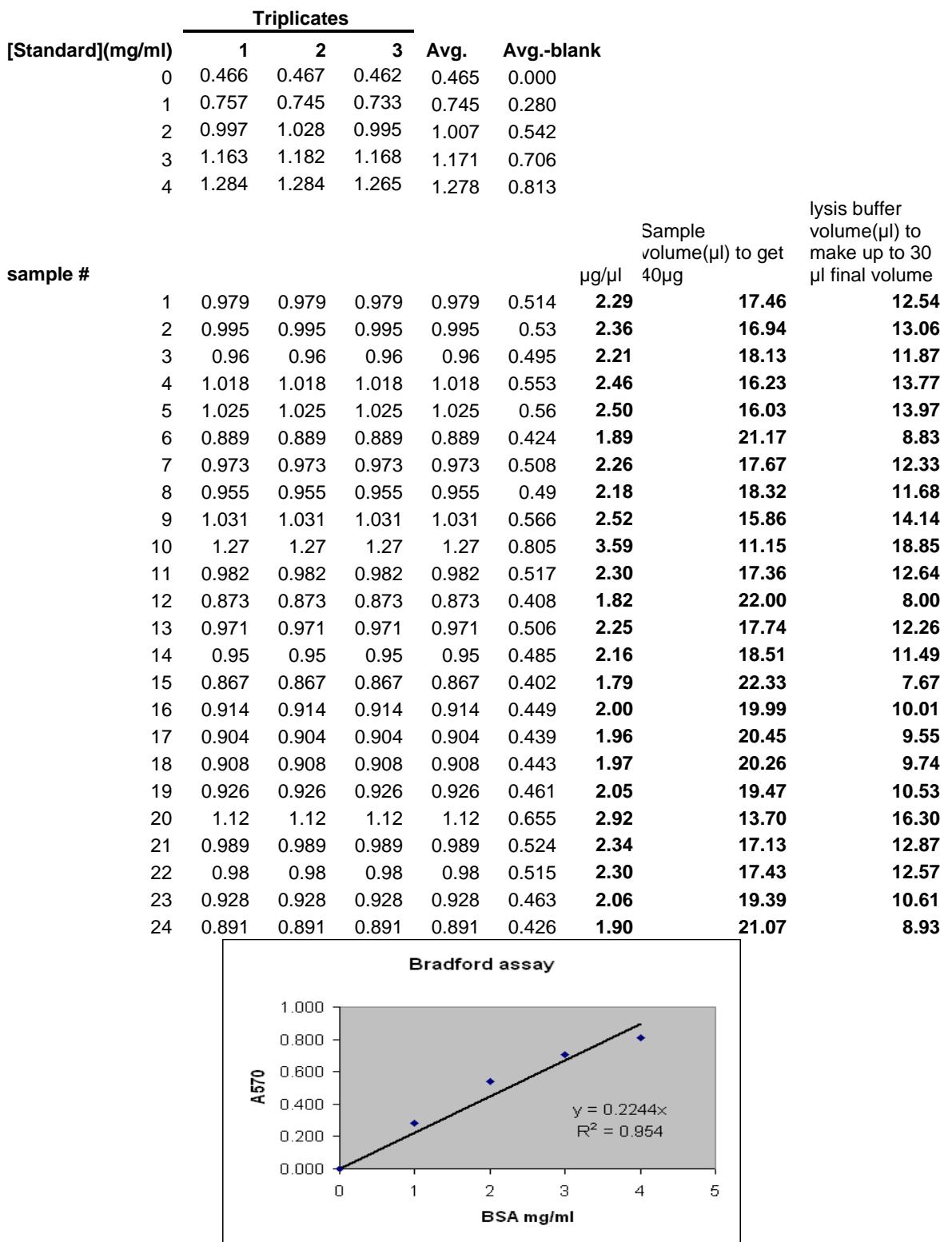
Frozen brain tissue was lysed in 40 $\mu$ l lysis buffer (80mM sodium  $\beta$ -glycerophosphate, 1mM dithiothreitol, 1mM sodium fluoride, pH to 7.6) containing protease inhibitor cocktail (1 $\mu$ l/1000 $\mu$ l of buffer) (Sigma–Aldrich Ireland, Ltd., Dublin, Ireland). Tissue was homogenised in a 1.5ml microcentrifuge tube using a pellet pestle cordless motor with polypropylene attachment (Sigma–Aldrich Ireland, Ltd., Dublin, Ireland) and centrifuged at 14,240g for 15min at 4°C. The supernatant was collected and Bradford assay (Bradford, 1976) was performed on 5 $\mu$ l supernatant as described below to determine protein levels. Samples were then diluted in ice-cold lysis buffer to give equal protein concentrations (40 $\mu$ g in 30 $\mu$ l of each sample) followed by addition of sample buffer (10 $\mu$ l) (50mM Tris–HCl, 1.84% SDS, 8% Glycerol, 2% bromophenol blue, and 5% 2-mercaptoethanol). Lysates were heated at 95°C for 5min. The proteins (40 $\mu$ g in 40 $\mu$ l of each sample) were then separated under reducing conditions by SDS–PAGE using 12% polyacrylamide gels (30% Protogel mix, 1.5M Tris (PH 8.8), 10% SDS, 10% ammonium persulfate, N,N, N', N'-tetramethylethylenediamine) and electroblotted onto a nitrocellulose protran membrane (0.2 $\mu$ m; Biorad laboratories, Medical supplies, Ireland). Membranes were rocked in blocking solution (5% milk, 0.1% Tween 20 in TBS) for 1hr. Separate membranes were then incubated in primary antibody diluent (5% milk, 0.1% Tween 20 in TBS) overnight at 4°C containing anti-Erk1/2 (1:5000) or anti-phospho- Erk1/2 (1:2000) (anti-pErk1/2 ,#9101; anti-Erk1/2,#9102; Cell Signaling Technologies, Boston, MA, USA). The following day, after three 5 minute washes in washing buffer, (0.25% Tween 20 in TBS), membranes were rocked at room temperature in secondary antibody solution (5% milk, 0.1% Tween 20 in TBS) containing secondary antibodies (1:2,000; peroxidase-conjugated AffiniPure mouse anti-rabbit IgG heavy and light antibodies, #211-032-171, Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK). Chemiluminescence and picture acquisition was performed under safe-light conditions. Membranes were exposed to chemiluminescent reagents (Super Signal West Dura Extended Duration Substrate, Thermo Fisher Scientific Inc., Rockford, IL, USA) for 5min followed by exposure to the G:BOX iChemi image analyzer (G:BOX iChemi image analyzer Syngene, UK ) for 5-20min

depending on the protein of interest. After acquiring the pictures for phospho-Erk1/2 and total Erk1/2, membranes were incubated in stripping buffer for 5min (25mM Glycine-HCl pH 2, 1%SDS) to remove the existing antibodies so that the membranes could be re-blotted for  $\beta$ -actin. After 3 x 5min in washing solution (0.25% Twen 20 in TBS), the same procedure was applied again to the membranes beginning with blocking, incubation with primary antibody solution containing anti- $\beta$ -Actin antibody (1:10,000) (Monoclonal mouse Anti- $\beta$ -Actin, #A5441; Sigma Aldrich, Dublin, Ireland) and the secondary antibody solution contained anti-mouse IgG (1:5000) (Anti-Mouse IgG (Fab specific)-Peroxidase goat antibody, #A3682; Sigma Aldrich, Ireland) and image analysis using the G-Box. The bands on all films were quantified using densitometric analysis on ImageJ (<http://www.rsb.info.nih.gov/ij/>) and presented (as in **Fig.2.11**) after inversion using inverted lookup table. Background integrated density values were computed and subsequently subtracted from band integrated density values to obtain corrected integrated density values. Corrected integrated density values of pErk1/2 and Erk1/2 were then normalised to their respective corrected integrated density of  $\beta$ -actin. The relative expression of pErk1/2 was obtained by dividing the normalised integrated density values for the pErk1/2 bands by the normalised integrated density value for the total Erk1/2 bands. In turn, these values for each treatment group were normalised to the control group (NFC-Veh) in each membrane and results were presented as percentage control value.

### **2.2.7 Bradford assay**

Bradford assay was used to determine the protein concentration of supernatants collected after 15min spin at 14,000g (described in section 2.2.6). Protein (BSA, Sigma Aldrich, Dublin, Ireland) standards (0, 1, 2, 3, 4mg/ml) were prepared in lysis buffer containing protease inhibitor. The Bradford assay consisted of adding 250ul Bradford reagent (Sigma Aldrich, Dublin, Ireland) to 5 $\mu$ l of unknown samples or standards (in triplicate) on a 96-well plate. After a 5min incubation time, absorption at 570nm wavelength was determined. Protein concentrations of the samples were determined using 5 point standard curve constructed using the BSA standards as shown in **Fig 2.3**.

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**Figure 2.3** Representative spreadsheet used for quantification and equalisation of protein concentrations with Bradford assay. Absorbance at 570nm for known standard concentrations fit to a line from which unknown sample concentrations could be calculated

### **2.2.8 Statistical analysis**

The SPSS 17.0 statistical package was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene test, respectively. Behavioural, neurochemical and western blotting data were analysed using two-factor analysis of variance (ANOVA), with the factors being fear-conditioning and formalin. Post-hoc pairwise comparisons were made with Fisher's LSD when appropriate. Data were expressed as group means  $\pm$  standard error of the mean ( $\pm$  SEM) and considered significant when  $P<0.05$ .

## **2.3 Results**

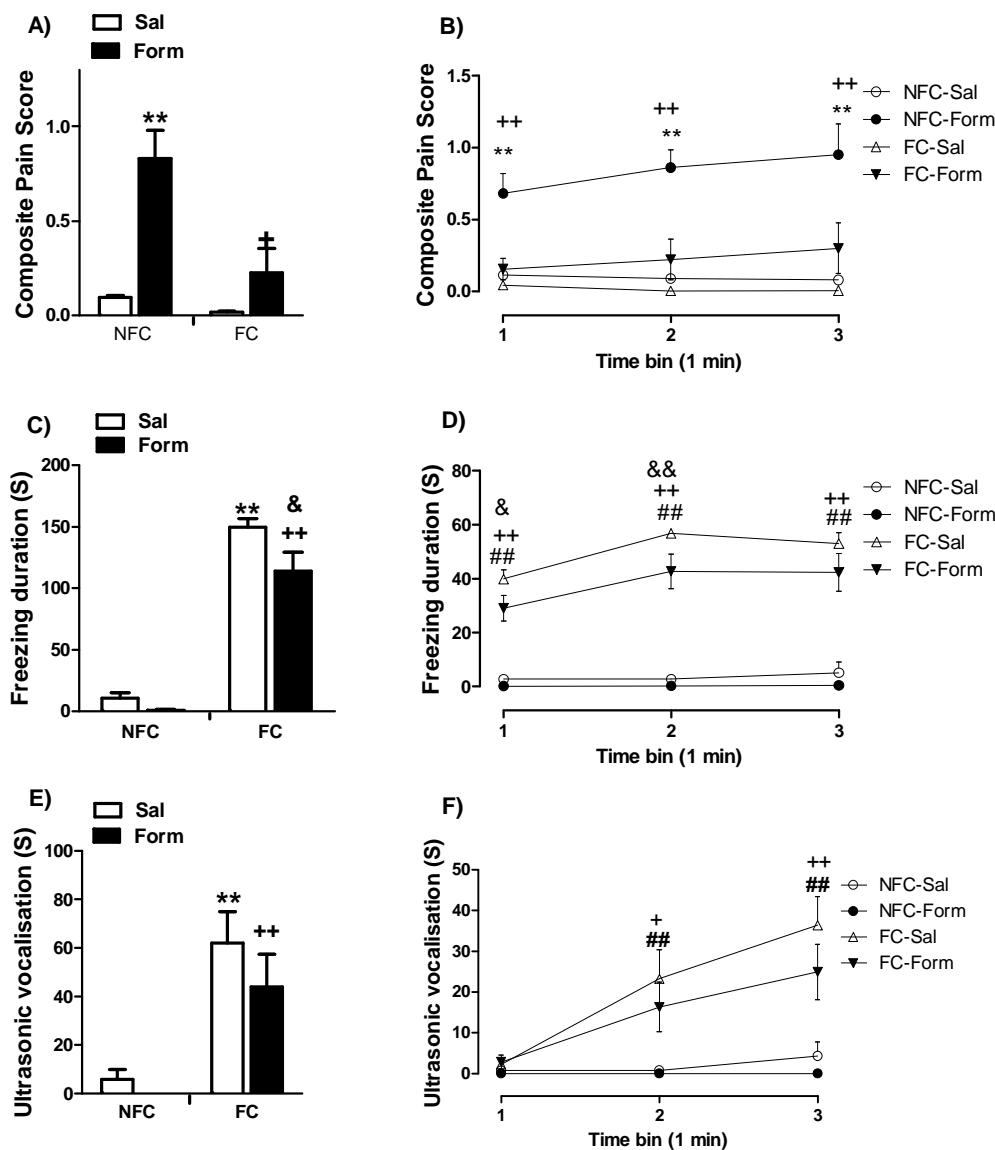
### ***2.3.1 Effects of fear-conditioning or/and intra-plantar formalin on nociceptive behaviour, expression of conditioned fear and FCA***

Non fear-conditioned rats receiving intra-plantar saline displayed little or no nociceptive behaviour or contextually-induced fear behaviour during the 3min trial period. In comparison, intra-plantar injection of formalin resulted in significant nociceptive responding (CPS) (NFC-Sal vs. NFC-Form,  $P < 0.01$ ; **Fig. 2.4A**) Contextual fear-conditioning resulted in an increase in the duration of freezing and 22kHz ultrasonic vocalisation in both saline- and formalin-injected rats when compared with non-fear -conditioned counterparts (NFC-Sal vs. FC-Sal,  $p < 0.01$ ; NFC-Form vs. FC-Form,  $p < 0.01$ ; **Fig. 2.4B&C**). Compared with formalin-injected non fear-conditioned rats, formalin-injected fear-conditioned rats showed a significant reduction in formalin-evoked nociceptive behaviour when re-exposed to the arena previously paired with footshock, confirming expression of FCA (CPS: NFC-Veh vs. FC-Veh,  $p < 0.01$ , **Fig. 2.4A**)

### ***2.3.2 Effects of intra-plantar formalin and fear-conditioning on hind paw oedema, general exploratory/locomotor behaviours and defecation***

Intra-plantar injection of formalin resulted in right hindpaw oedema in non-fear conditioned and fear-conditioned rats (NFC-Sal vs. NFC-Form 1,  $p < 0.01$ ; FC-Sal vs. FC-Form,  $p < 0.05$ ; **Table 2.1**). In non fear-conditioned rats, intra-plantar administration of formalin significantly reduced general locomotor activity (total activity) measured as the sum of time spent rearing, grooming and walking when compared with saline-treated rats (NFC-Sal vs. NFC-Form,  $p < 0.01$ ; **Table 2.1**). Similarly, fear conditioning was associated with a significant reduction in the duration of total activity in both saline- and formalin-treated rats (NFC-Sal vs. FC-Sal,  $p < 0.01$ ; NFC-Form vs. FC-Form,  $p < 0.05$ ; **Table 2.1**). Fear conditioning also resulted in significant a increase in defecation in both saline- and formalin-treated rats (NFC-Sal vs. FC-Sal,  $p < 0.01$ ; NFC-Form vs. FC-Form,  $p < 0.05$ ; **Table 2.1**)

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**Figure 2.4** Effects of fear conditioning and intra-plantar formalin, alone or in combination, on A) formalin-evoked nociceptive behaviour (ANOVA: formalin:  $F_{(1, 44)}=22.77$ ,  $p<0.01$ ; fear-conditioning:  $F_{(1, 44)}=11.85$ ,  $p<0.01$  and formalin x fear-conditioning interaction:  $F_{(1, 44)}=7.13$ ,  $p<0.05$ ), B) 1min time bin presentation of CPS C) duration of freezing (ANOVA: fear-conditioning:  $F_{(1, 44)}=209.62$ ,  $p<0.01$ ; formalin:  $F_{(1, 44)}=6.83$ ,  $p<0.05$ ; formalin x fear conditioning interaction:  $F_{(1, 44)}=2.21$ ,  $p=0.14$ ) and D) 1min time bin presentation of duration of freezing E) duration of 22kHz ultrasonic vocalisation (ANOVA: fear-conditioning  $F_{(1, 44)}=27.50$ ,  $p<0.01$ ; formalin:  $F_{(1, 44)}=1.55$ ,  $p=0.22$ ; formalin x fear conditioning interaction:  $F_{(1, 44)}=.39$ ,  $p=0.54$ ) F) 1min time bin presentation of duration of ultrasonic vocalisation over the 3min trial period; \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal; + $p<0.05$ , ++ $p<0.01$  vs. NFC-Form, & $p<0.05$  vs. FC- Sal and 1min time bin (D,E,F) presentation of these behaviours, respectively, \*\* $p<0.01$  NFC-Sal vs. NFC-Form; ## $p<0.01$  NFC-Sal vs. FC-Sal; + $p<0.05$ , ++ $p<0.01$  NFC-Form vs. FC-Form; & $p<0.05$ , && $p<0.01$  FC-Sal vs. FC-Form. Data are expressed in mean+SEM ( $n=12$ ); FC, fear-conditioned; NFC, non-fear-conditioned; Sal, Saline; Form, Formalin

Groups	Paw diameter (mm)		Total activity (S)		Defecation ( number of pellets )	
	Mean	±SEM	n	±SEM	Mean	±SEM
NFC-Sal	0.91	±0.09	48.17	±5.17	1.25	±0.28
NFC-Form	1.66	±0.07**	27.92	±4.05**	1	±0.49
FC-Sal	0.85	±0.07	6.92	±2.58**	3.5	±0.31**
FC-Form	1.63	±0.07#	7.00	±1.73 <sup>+</sup>	2.91	±0.43 <sup>+</sup>

**Table 2.1** Effects of fear conditioning and intra-plantar formalin, alone or in combination, on general exploratory/locomotor behaviours, hind paw oedema and defecation; Two way ANOVA for total activity revealed: fear conditioning:  $F_{(1,44)}=73.17$ , p<0.01, formalin:  $F_{(1,44)}=7.7$ , p<0.01, fear conditioning x formalin,  $F_{(1,44)}=7.83$ , p=0.08; Hind paw oedema: fear conditioning;  $F_{(1,44)}=0.003$  p=0.957, formalin;  $F_{(1,44)}=96.3$ , p<0.01, fear conditioning x formalin  $F_{(1,44)}=0.141$ , p=0.71 ; Defecation: fear conditioning;  $F_{(1,44)}=28.59$ , p<0.01, formalin;  $F_{(1,44)}=1.143$ , p= 0.29, fear conditioning x formalin  $F_{(1,44)}=0.183$ , p=0.67; \*\*p<0.01 vs. NFC-Sal, +p<0.05 vs. NFC-Form, #p<0.05 vs. FC-Sal (ANOVA followed by Fisher's LSD posthoc test); Data are mean±SEM (n=12); FC, fear-conditioned; NFC, non fear-conditioned; Sal, Saline; Form, Formalin

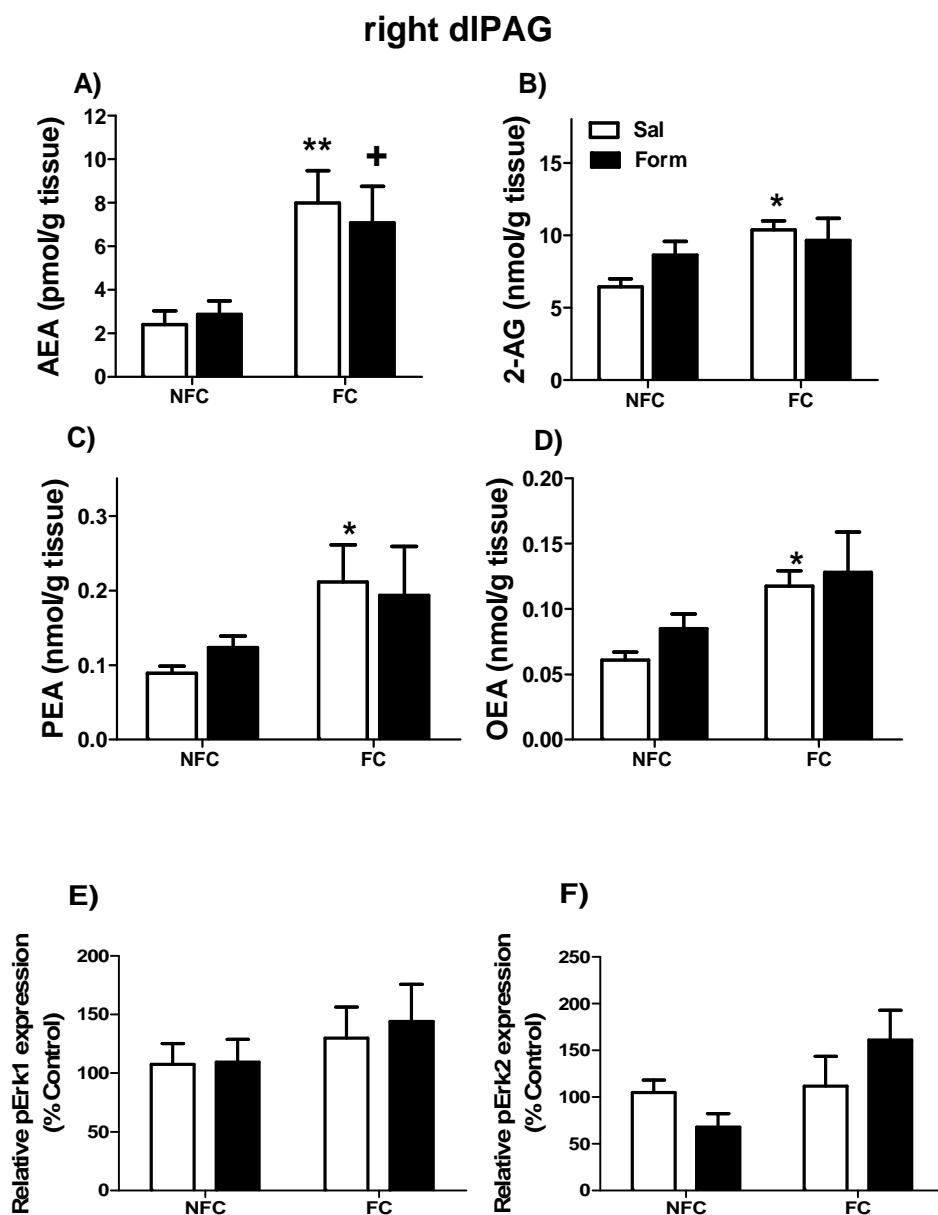
### *2.3.3 Effect of fear-conditioning and intra-plantar formalin on levels of endocannabinoids and NAEs in discrete brain regions*

In addition to the tables (Table 2.2 and Table 2.3) presenting the endocannabinoid/NAE and relative pErk data for all regions investigated, results pertaining to the PAG, BLA and RVM, where statistically significant changes in levels of endocannabinoids or pErk1/2 were found, are also presented in graph form in Figs 2.5-2.10. The findings demonstrate that in non-fear-conditioned rats, formalin injection did not affect levels of endocannabinoids/NAEs in the dlPAG, vIPAG, BLA, RVM, or hippocampus; but decreased levels of AEA in left lateral PAG, decreased levels of 2-AG in the right lateral PAG, increased levels of AEA in the left insular cortex and increased 2-AG and PEA in the PFC, compared with non fear-conditioned saline-treated rats (NFC-Sal vs. NFC- Form, **Fig 2.5-2.10 & Table 2.2**). In contrast, in fear-conditioned rats, formalin injection increased levels of AEA in the left lateral PAG, decreased levels of 2-AG in both right and left vIPAG, decreased 2-AG in left dlPAG and decreased 2-AG in the RVM, compared with saline-injected controls (FC-Sal vs. FC- Form, **Fig 2.5-2.10 & Table 2.2**). Formalin injection in fear-conditioned rats had no

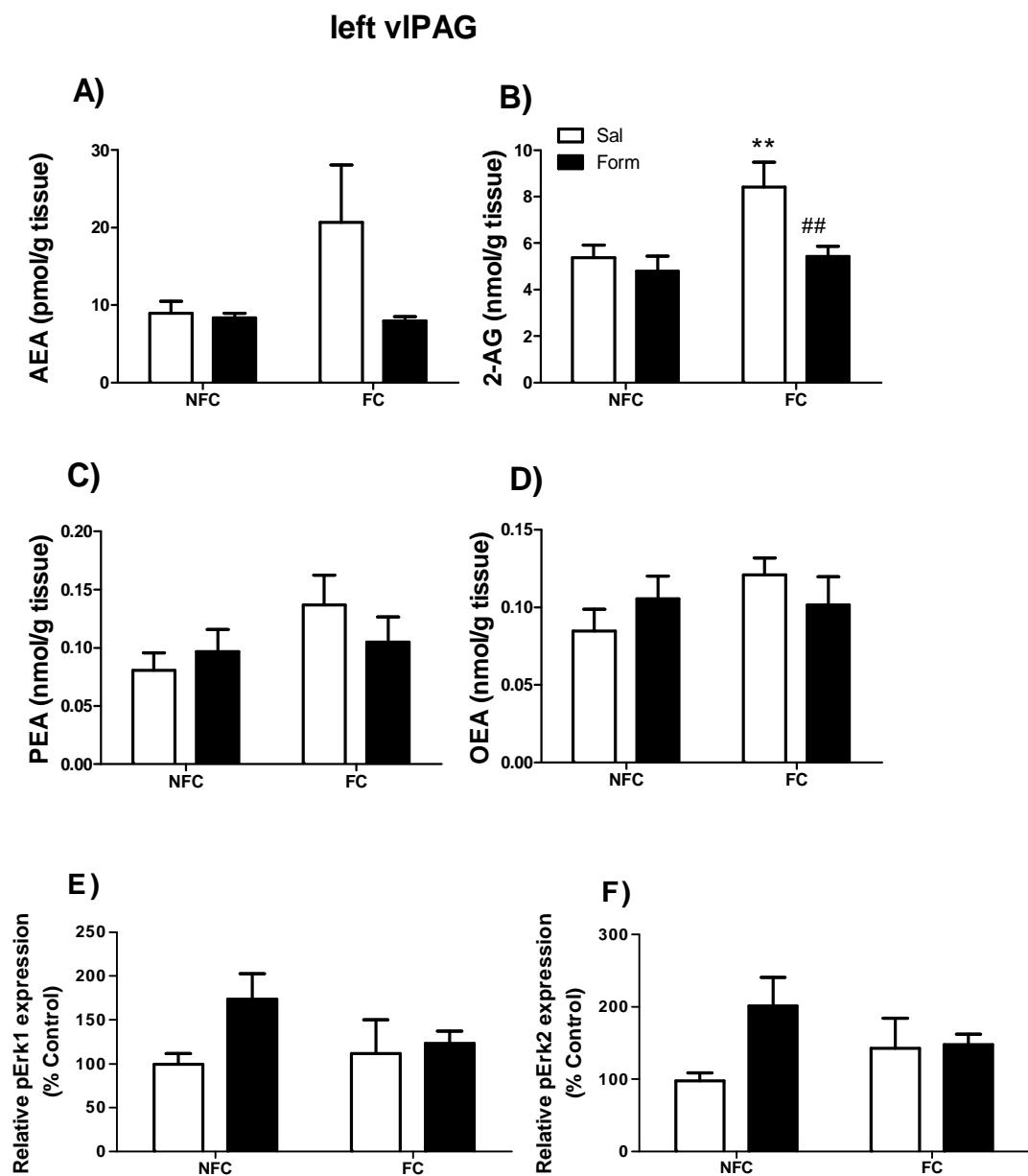
significant effect on levels of endocannabinoids or NAEs in the dlPAG, BLA, hippocampus, insula and PFC.

Fear-conditioned rats receiving intra-plantar saline showed increased levels of all analytes in the right dlPAG, increased levels of PEA in the left dlPAG, increased levels of 2-AG in both right and left vIPAG, but decreased levels of AEA in the left lateral PAG when compared with non-fear-conditioned saline-treated rats (NFC-Sal vs FC-Sal, **Fig. 2.5-2.8 & Table 2.2**). In addition, fear conditioning was associated with increased levels of AEA, PEA and OEA in the left BLA, increased levels of OEA in the right BLA, increased AEA and 2-AG content in the RVM and increased PEA in the left dorsal hippocampus (NFC-Sal vs. FC-Sal, **Fig. 2.8-2.10 & Table 2.2**).

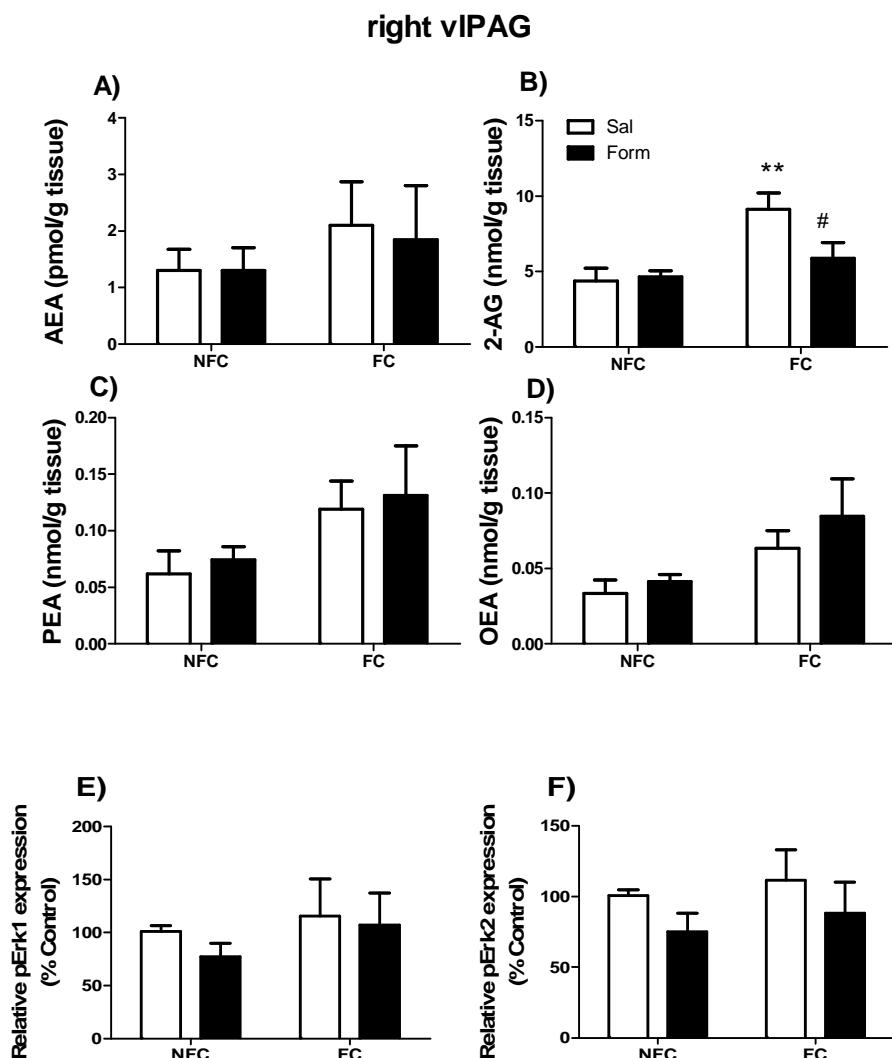
In the presence of formalin-induced nociceptive tone, contextual fear conditioning suppressed nociceptive behaviour as mentioned above (i.e. induced FCA) and resulted in increased levels of AEA in the right dlPAG (with a strong trend for increased levels of PEA and OEA) but decreased levels of 2-AG in the left dlPAG when compared with non-fear-conditioned formalin-treated rats (NFC-Form vs. FC-Form, **Fig 2.5 & Table 2.2**). FCA was also accompanied by increased levels of AEA in the left lateral PAG (NFC-Form vs. FC-Form, **Table 2.2**).



**Figure 2.5** AEA, 2-AG, PEA and OEA levels (A-D) and Relative pErk1/2 expression (E-F) in the right dlPAG in rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia. A) AEA (ANOVA: Fear-conditioning:  $F_{(1, 20)}=16.50$ ,  $p<0.01$ ; formalin:  $F_{(1, 20)}=0.03$ ,  $p=0.85$ ; fear conditioning x formalin:  $F_{(1, 20)}=0.33$ ,  $p=0.57$ ). B) 2-AG (ANOVA: fear conditioning:  $F_{(1, 20)}=6.168$ ,  $p<0.05$ ; form:  $F_{(1, 20)}=0.557$ ,  $p=0.464$ ; form x fear conditioning  $F_{(1, 20)}=2.201$ ,  $p=0.154$ ). C) PEA (ANOVA: fear conditioning  $F_{(1, 20)}=6.20$ ,  $p<0.05$ ; form:  $F_{(1, 20)}=0.00$ ,  $p=0.99$ ; fear conditioning x form:  $F_{(1, 20)}=0.18$ ,  $p=0.67$ ). D) OEA (ANOVA: fear conditioning:  $F_{(1, 20)}=7.82$ ,  $p<0.05$ ; form:  $F_{(1, 20)}=0.94$ ,  $p=0.34$ ; fear conditioning x form:  $F_{(1, 20)}=0.13$ ,  $p=0.71$ ). E) relative pErk1 F) relative pErk2. \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, + $p<0.05$ , ++ $p<0.01$  vs. NFC-Form, #  $p<0.05$  vs. FC-Sal (Fisher's LSD). Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). FC, fear-conditioned; NFC, non fear-conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

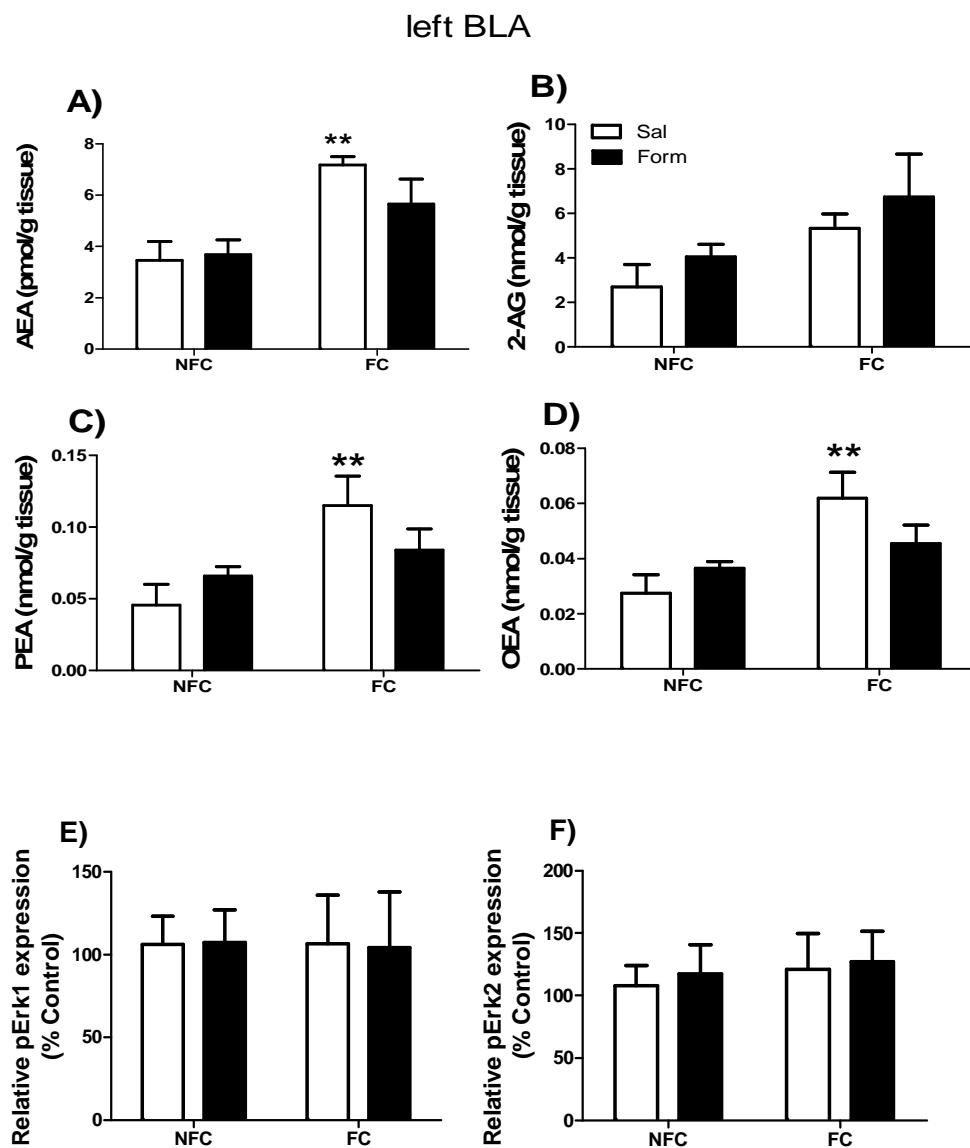


**Figure 2.6** AEA, 2-AG, PEA and OEA levels (A-D) and Relative pErk1/2 expression (E&F) in the left vIPAG in rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia A) AEA (ANOVA: fear conditioning:  $F_{(1, 16)}=2.21$ ,  $p=0.15$ ; formalin:  $F_{(1, 16)}=0.3.07$ ,  $p=0.09$ ; fear conditioning x formalin:  $F_{(1, 16)}=0.535$ ,  $p=0.13$  B) 2-AG (ANOVA: fear conditioning:  $F_{(1, 16)}=6.65$ ,  $p<0.05$ ; form:  $F_{(1, 16)}=0.6.29$ ,  $p<0.05$ ; form x fear conditioning  $F_{(1, 16)}=2.90$ ,  $p=0.11$  C) PEA (ANOVA: fear conditioning  $F_{(1,16)}=1.66$ ,  $p=0.21$ ) form:  $F_{(1, 16)}=0.49$ ,  $p=0.49$ ; form x fear conditioning  $F_{(1, 16)}=0.77$ ,  $p=0.39$  D) OEA (ANOVA: fear conditioning:  $F_{(1, 16)}=0.94$ ,  $p=0.34$ ; form:  $F_{(1, 16)}=0.004$ ,  $p=0.09$ ; fear conditioning x form:  $F_{(1, 16)}= 2.21$ ,  $p=0.15$ ) E) relative pErk1: F) relative pErk 2; \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, + $p<0.05$ , ++ $p<0.01$  vs. NFC-Form, # $p<0.05$  vs. FC-Sal (Fisher's LSD); Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). FC, fear-conditioned; NFC, non-fear conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, *N*-palmitoyl ethanolamide; OEA, *N*-oleoyl ethanolamide.

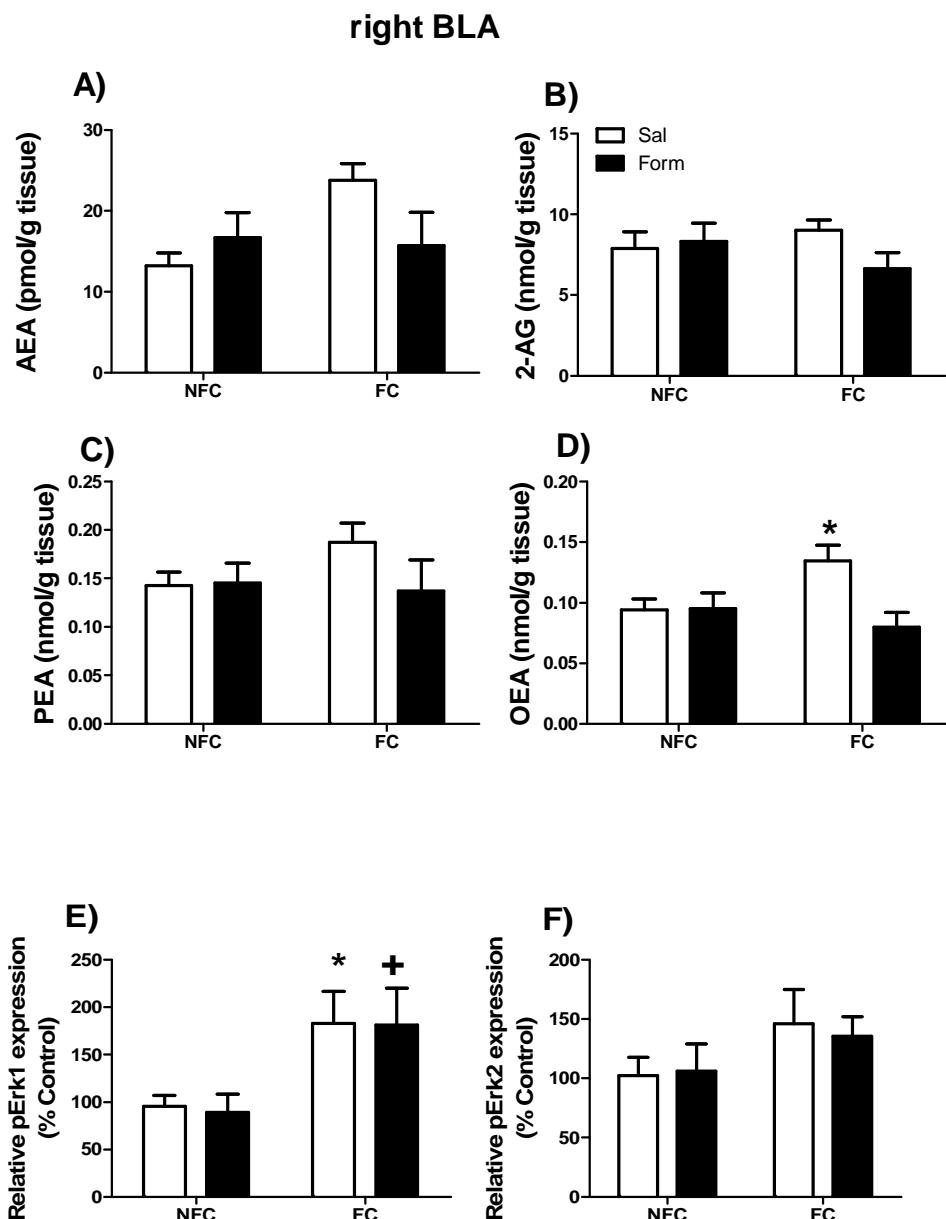


**Figure 2.7** AEA, 2-AG, PEA and OEA levels (A-D) and Relative pErk1/2 expression (E&F) in the right vIPAG in rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia. A) AEA (ANOVA: fear conditioning:  $F_{(1, 17)}=0.93$ ,  $p=0.86$ ; formalin:  $F_{(1, 17)}=0.03$ ,  $p=0.86$ ; fear conditioning x formalin:  $F_{(1, 17)}=0.03$ ,  $p=0.85$ ). B) 2-AG (ANOVA: fear conditioning:  $F_{(1, 18)}=0.89$ ,  $p=0.11$ ; form:  $F_{(1, 18)}=11.87$ ,  $p<0.01$ ; form x fear conditioning  $F_{(1, 18)}=4.08$ ,  $p=0.059$ ). C) PEA (ANOVA: fear conditioning  $F_{(1, 16)}=0.20$ ,  $p=0.65$ ; form:  $F_{(1, 16)}=4.26$ ,  $p=0.056$ ; fear conditioning x form:  $F_{(1, 16)}=0.00$ ,  $p=0.99$ ). D) OEA (ANOVA: fear conditioning:  $F_{(1, 17)}=0.80$ ,  $p=0.63$ ; form:  $F_{(1, 17)}=5.06$ ,  $p<0.05$ ; fear conditioning x form:  $F_{(1, 17)}=0.17$ ,  $p=0.60$ ). E) relative pErk1 F) relative pErk2. \*\* $p<0.01$  vs. NFC-Sal, #  $p<0.05$  vs. FC-Sal (Fisher's LSD). Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). FC, fear-conditioned; NFC, non-fear-conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

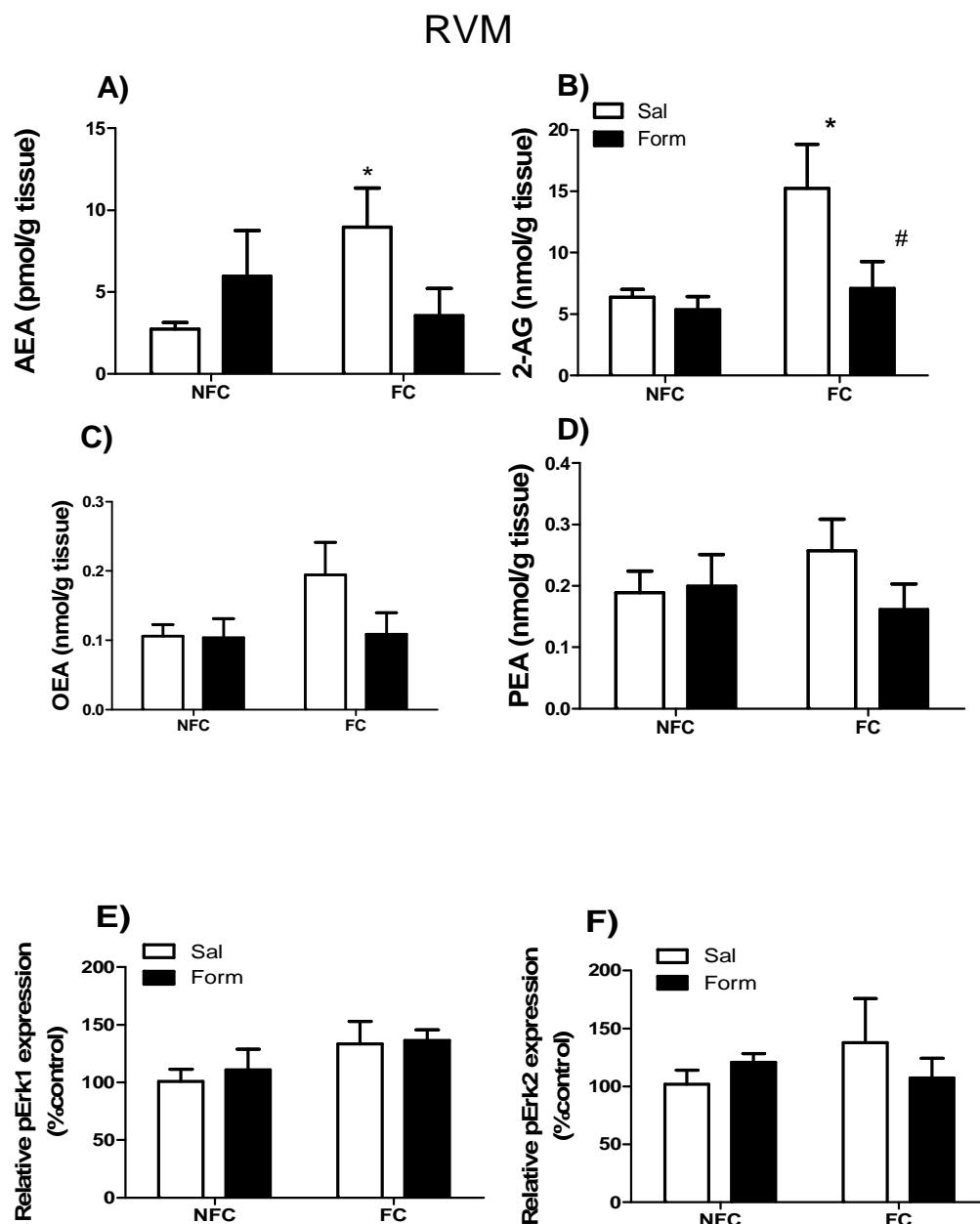
**Chapter 2: Characterisation of brain regional levels of endocannabinoids and N-acylethanalamines during expression of conditioned fear, pain or fear-conditioned analgesia in rats**



**Figure 2.8** AEA, 2-AG, PEA and OEA levels (A-D) and Relative pErk1/2 expression (E&F) in the left BLA in rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia. A) AEA (ANOVA: fear conditioning:  $F_{(1, 17)}=14.9$ ,  $p=0.001$ ; formalin:  $F_{(1, 17)}=0.79$ ,  $p=0.39$ ; fear conditioning x formalin:  $F_{(1, 17)}=1.39$ ,  $p=0.26$ ). B) 2-AG (ANOVA: fear conditioning:  $F_{(1, 19)}=4.99$ ,  $p<0.05$ ; form:  $F_{(1, 19)}=1.35$   $p=0.26$ ; form x fear conditioning  $F_{(1, 19)}=0.00$ ,  $p=0.98$ ). C) PEA (ANOVA: fear conditioning  $F_{(1, 19)}=8.42$ ,  $p<0.01$ ; form:  $F_{(1, 19)}=0.13$ ,  $p=0.72$ ; fear conditioning x form:  $F_{(1, 19)}=0.10$ ,  $p=0.10$ ). D) OEA (ANOVA: fear conditioning:  $F_{(1, 19)}=10.12$ ,  $p<0.01$ ; form:  $F_{(1, 19)}=0.29$ ,  $p=0.59$ ; fear conditioning x form:  $F_{(1, 19)}=3.52$ ,  $p=0.07$ ). E) relative pErk1 F) relative pErk2 \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, + $p<0.05$ , ++ $p<0.01$  vs. NFC-Form, #  $p<0.05$  vs. FC-Sal (Fisher's LSD). Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). FC, fear-conditioned; NFC, non-fear-conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, *N*-palmitoyl ethanolamide; OEA, *N*-oleoyl ethanolamide.



**Figure 2.9** AEA, 2-AG, PEA and OEA levels (A-D) and Relative pErk1/2 expression (E-F) in the right BLA in rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia. A) AEA (ANOVA: fear conditioning:  $F_{(1, 20)}=2.76$ ,  $p=0.11$ ; formalin:  $F_{(1, 20)}=0.63$ ,  $p=0.43$ ; fear conditioning x formalin:  $F_{(1, 20)}=4.094$ ,  $p=0.57$ ) B) 2-AG (ANOVA: fear conditioning:  $F_{(1, 19)}=0.08$ ,  $p=0.76$ ; form:  $F_{(1, 19)}=1.03$ ,  $p=0.32$ ; form x fear conditioning  $F_{(1, 19)}=2.18$ ,  $p=0.15$ ) C) PEA (ANOVA: fear conditioning  $F_{(1, 20)}=0.63$ ,  $p=0.43$ ); form:  $F_{(1, 20)}=1.10$ ,  $p=0.30$ ; form x fear conditioning  $F_{(1, 20)}=1.33$ ,  $p=0.26$ ) D) OEA (ANOVA: fear conditioning:  $F_{(1, 20)}=1.14$ ,  $p=0.29$ ; form:  $F_{(1, 20)}=5.25$ ,  $p=0.03$ ; fear conditioning x form:  $F_{(1, 20)}=5.6$ ,  $p<0.05$ ) E) relative pErk1: F) relative pErk2 \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, + $p<0.05$ , ++ $p<0.01$  vs. NFC-Form, # $p<0.05$  vs. FC-Sal (Fisher's LSD); Data expressed as Mean  $\pm$  SEM (n=5-6). FC, fear-conditioned; NFC, non-fear-conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.



**Figure 2.10** AEA, 2-AG, PEA and OEA levels (A-D) and Relative pErk1/2 expression (E&F) in the RVM in rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia. A) AEA (ANOVA: Fear-conditioning:  $F_{(1, 20)}=0.87$ ,  $p=0.36$ ; formalin:  $F_{(1, 20)}=0.29$ ,  $p=0.59$ ; fear-conditioning x formalin:  $F_{(1, 20)}=4.55$ ,  $p<0.05$ ; B) 2-AG (ANOVA: fear-conditioning:  $F_{(1, 20)}=4.19$ ,  $p<0.05$ ; form:  $F_{(1, 20)}=3.66$ ,  $p=0.07$ ; fear-conditioning x form:  $F_{(1, 20)}=2.19$ ,  $p=0.15$ ) C) PEA (ANOVA: fear-conditioning  $F_{(1, 17)}=0.09$ ,  $p=0.76$ ) form:  $F_{(1, 17)}=0.09$ ,  $p=0.76$ ; fear-conditioning x form:  $F_{(1, 17)}=1.30$ ,  $p=0.27$ )D) OEA (ANOVA: fear-conditioning:  $F_{(1, 16)}=2.09$ ,  $p=0.16$ ; form:  $F_{(1, 16)}=1.86$ ,  $p=0.19$ ; fear-conditioning x form:  $F_{(1, 16)}=1.67$ ,  $p=0.21$ ) E) perk2: F) perk 1 \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, + $p<0.05$ , ++ $p<0.01$  vs. NFC-Form, # $p<0.05$  vs. FC-Sal (Fisher's LSD); Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). FC, fear-conditioned; NFC, non fear conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, *N*-palmitoyl ethanolamide; OEA, *N*-oleoyl ethanolamide.

**Table 2.2 Levels of AEA (pmol/g), 2-AG, PEA and OEA (nmol/g) in discrete brain regions of rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia**

Region	Side	Analyte	NFC-Sal	NFC-Form	FC-Sal	FC-Form	Fear conditioning (F, DF, P values)	Form (F, DF, P values)	Interaction (F, DF, P values)
dlPAG	right	<b>AEA</b>	2.87 ±0.5	3.44 ±0.3	7.99 ±3.26**	7.07± 2.89+	F 1,20= 16.5, 0.001	F 1,20=0.035, 0.853	F 1,20=0.332, 0.571
		<b>2-AG</b>	6.42 0.57	8.63 0.93	10.35± 0.61*	9.62 ±1.54	F 1,20=6.17, 0.02	F 1,20=0.55, 0.46	F 1,20=2.2, 0.15
		<b>PEA</b>	0.08 ±0.01	0.11 ±0.02	0.21 ±0.05*	0.19 ±0.07	F 1,20=6.20, 0.02	F 1,20=0.00, 0.99	F 1,20=0.18, 0.67
		<b>OEA</b>	0.06 ±0.01	0.08 ±0.01	0.12 ±0.01*	0.13 ±0.03	F 1,20=7.83, 0.01	F 1,20=0.94, 0.34	F 1,20=0.13, 0.71
	left	<b>AEA</b>	21.74± 3.09	24.34 ±5.59	24.33± 2.96	22.88 ±4.48	F 1,20=0.655, 0.89	F 1,20=0.01, 0.98	F 1,20=2.70, 0.63
		<b>2-AG</b>	10.48 ±0.95	12.15 ±1.04	11.55 ±0.83	7.92 ±0.91#++	F 1,20=2.87, 0.10	F 1,20=1.11, 0.30	F 1,20=8.06, 0.01
		<b>PEA</b>	0.14 ±0.01	0.17 ±0.01	0.18 ±0.02*	0.16 ±0.01	F 1,20=0.80, 0.38	F 1,20=0.12, 0.72	F 1,20=5.01, 0.03
		<b>OEA</b>	0.13 ±0.01	0.15 ±0.01	0.16 ±0.02	0.12 ±0.01#	F 1,20=0.08, 0.78	F 1,20=0.42, 0.52	F 1,20=4.76, 0.04
vlPAG	right	<b>AEA</b>	1.30 ±0.37	1.30 ±0.4	2.10 ±0.77	1.84 ±0.96	F 1,17=0.93, 0.35	F 1,17=0.03, 0.85	F 1,17=0.03, 0.85
		<b>2-AG</b>	5.06 ±0.97	4.65 ±0.4	8.01 ±1.41**	5.89 ±1.02##	F 1,18=2.89, 0.10	F 1,18=11.87, 0.003	F 1,18=4.07, 0.059
		<b>PEA</b>	0.09 ±0.03	0.09±0.02	0.21 ±0.09	0.17 ±0.05	F 1,16=0.20, 0.65	F 1,16=4.26, 0.056	F 1,16=0.00, 0.99
		<b>OEA</b>	0.05 ±0.01	0.05±0.01	0.11 ±0.05	0.08 ±0.02	F 1,17=0.80, 0.63	F 1,17=5.06, 0.03	F 1,17=0.16, 0.60
	left	<b>AEA</b>	8.95 ±1.56	8.33 ±0.61	20.68 ±7.41	7.94 ±0.56	F 1,16=2.21, , 0.15	F 1,16=3.07, 0.09	F 1,16=2.53, 0.13
		<b>2-AG</b>	5.36 ±0.55	4.79 ±0.64	8.42 ±1.06**	5.41 ±0.44##	F 1,16=6.65, 0.02	F 1,16=6.29, 0.02	F 1,16=2.90, 0.10
		<b>PEA</b>	0.09 ±0.02	0.09 ±0.02	0.16 ±0.03	0.10 ±0.02	F 1,20=1.66, 0.21	F 1,20=0.49, 0.49	F 1,20=0.77, 0.39
		<b>OEA</b>	0.09 ±0.02	0.09 ±0.02	0.14 ±0.02	0.10 ±0.02	F 1,18=0.94, 0.34	F 1,18=0.00, 0.94	F 1,18=2.21, 0.15
lateral PAG	left	<b>AEA</b>	10.56 ±1.18	4.51 ±0.52**	5.63 ±1.99*	11.99 ±2.12+#+	F 1,17=0.13, 0.72	F 1,17=0.17, 0.68	F 1,17=14.53, 0.00
		<b>2-AG</b>	3.13 ±0.6	2.65 ±0.29	3.41 ±0.67	5.64 ±2.73	F 1,17=0.32, 0.57	F 1,17=0.03, 0.84	F 1,17=0.03, 0.85
		<b>PEA</b>	0.11 ±0.02	0.09 ±0.01	0.12 ±0.02	0.15 ±0.04	F 1,20=1.23, 0.27	F 1,20=0.09, 0.76	F 1,20=1.03, 0.32
		<b>OEA</b>	0.12 ±0.02	0.09 ±0.01	0.12 ±0.02	0.14 ±0.03	F 1,17=0.82, 0.37	F 1,17=0.04, 0.83	F 1,17=0.15, 0.70
	right	<b>AEA</b>	16.11±4.77	9.10 ±2.9	11.96 ±2.42	19.96 ±5.22	F 1,20=1.95, 0.18	F 1,20=0.01, 0.93	F 1,20=3.74, 0.07
		<b>2-AG</b>	5.86 ±1.33	4.03 ±0.86*	4.89 ±0.47	6.36 ±1.07	F 1,19=0.01, 0.91	F 1,19=0.36, 0.36	F 1,19=0.01, 0.01
		<b>PEA</b>	0.16 ±0.04	0.11 ±0.02	0.14 ±0.02	0.21 ±0.04	F 1,19=2.02, 0.17	F 1,19=0.00, 0.99	F 1,19=3.87, 0.06
		<b>OEA</b>	0.18 ±0.05	0.14 ±0.04	0.16 ±0.02	0.22 ±0.05	F 1,17=0.08, 0.77	F 1,17=0.25, 0.68	F 1,17=0.53, 0.47

**Table 2.2 Continued**

Regions	Side	Analyte	NFC-Sal	NFC-Form	FC-Sal	FC-Form	Fear conditioning (F, DF, P values)	Form (F, DF, P values)	Interaction (F, DF, P values)
vHipp	left	<b>AEA</b>	60.06 ± 5.30	50.94 ± 5.98	59.83 ± 13.61	71.25 ± 20.02	F 1,19=0.09, 0.76	F 1,19=0.48, 0.49	F 1,19=0.04, 0.82
		<b>2-AG</b>	9.22 ± 1.47	13.87 ± 3.07	9.48 ± 1.75	12.15 ± 2.00	F 1,18=0.01, 0.94	F 1,18=0.96, 0.34	F 1,18=0.44, 0.51
		<b>PEA</b>	0.13 ± 0.01	1.11 ± 0.65	0.18 ± 0.05	0.19 ± 0.07	F 1,20=1.69, 0.20	F 1,20=2.32, 0.14	F 1,20=2.13, 0.16
		<b>OEA</b>	0.13 ± 0.01	0.39 ± 0.25	0.15 ± 0.03	0.17 ± 0.04	F 1,17=0.00, 0.98	F 1,17=0.02, 0.89	F 1,17=0.93, 0.34
	right	<b>AEA</b>	57.56 ± 6.53	57.16 ± 8.89	49.54 ± 5.19	46.57 ± 8.51	F 1,16=2.94, 0.10	F 1,16=1.09, 0.31	F 1,16=0.10, 0.74
		<b>2-AG</b>	8.44 ± 0.87	9.76 ± 1.51	9.52 ± 0.58	9.35 ± 1.27	F 1,16=0.10, 0.75	F 1,16=1.64, 0.21	F 1,16=0.52, 0.47
		<b>PEA</b>	0.13 ± 0.01	0.18 ± 0.05	0.14 ± 0.03	0.13 ± 0.03	F 1,17=0.01, 0.91	F 1,17=0.39, 0.53	F 1,17=2.33, 0.14
		<b>OEA</b>	0.13 ± 0.02	0.13 ± 0.01	0.10 ± 0.01	0.11 ± 0.02	F 1,16=37, 0.07	F 1,16=0.00, 0.99	F 1,16=0.23, 0.63
dHipp	left	<b>AEA</b>	43.02 ± 4.28	40.12 ± 2.96	49.21 ± 8.05	42.15 ± 4.35	F 1,20=0.60, 0.44	F 1,20=0.89, 0.35	F 1,20=0.15, 0.69
		<b>2-AG</b>	5.41 ± 0.47	8.08 ± 1.06	6.99 ± 0.96	7.44 ± 1.32	F 1,20=0.54, 0.46	F 1,20=1.56, 0.21	F 1,20=1.89, 0.18
		<b>PEA</b>	0.16 ± 0.01	0.20 ± 0.02	0.24 ± 0.03*	0.19 ± 0.03	F 1,20=2.19, 0.10	F 1,20=0.01, 0.91	F 1,20=3.99, 0.05
		<b>OEA</b>	0.127 ± 0.01	0.145 ± 0.01	0.16 ± 0.01	0.14 ± 0.02	F 1,20=1.51, 0.23	F 1,20=0.01, 0.89	F 1,20=1.99, 0.17
	right	<b>AEA</b>	38.21 ± 4.20	38.25 ± 4.09	39.40 ± 4.84	42.53 ± 6.73	F 1,18=0.27, 0.60	F 1,18=0.09, 0.76	F 1,18=0.08, 0.77
		<b>2-AG</b>	11.85 ± 7.55	5.08 ± 0.55	5.79 ± 1.08	4.86 ± 0.58	F 1,19=0.01, 0.42	F 1,19=0.68, 0.91	F 1,19=1.24, 0.27
		<b>PEA</b>	0.21 ± 0.05	0.18 ± 0.01	0.18 ± 0.02	0.18 ± 0.03	F 1,19=0.20, 0.65	F 1,19=0.09, 0.75	F 1,19=0.72, 0.72
		<b>OEA</b>	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	F 1,19=0.05, 0.81	F 1,19=0.26, 0.61	F 1,19=0.52, 0.48
Insula	left	<b>AEA</b>	39.74 ± 1.47	49.79 ± 1.71*	52.16 ± 4.99	45.16 ± 3.56	F 1,20=1.42, 0.24	F 1,20=0.22, 0.64	F 1,20=6.81, 0.01
		<b>2-AG</b>	2.49 ± 0.4	2.23 ± 0.21	2.48 ± 0.41	2.68 ± 0.32	F 1,19=0.42, 0.52	F 1,19=0.01, 0.94	F 1,19=0.47, 0.49
		<b>PEA</b>	0.11 ± 0.00	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	F 1,20=3.20, 0.08	F 1,20=0.59, 0.44	F 1,20=2.36, 0.14
		<b>OEA</b>	0.14 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	F 1,20=1.69, 0.20	F 1,20=1.39, 0.25	F 1,20=3.54, 0.07
	right	<b>AEA</b>	57.31 ± 4.61	57.49 ± 2.23	58.72 ± 6.20	49.87 ± 4.01	F 1,20=0.47, 0.49	F 1,20=0.92, 0.34	F 1,20=1.00, 0.32
		<b>2-AG</b>	2.53 ± 0.39	1.68 ± 0.37	2.91 ± 0.83	1.73 ± 0.17	F 1,20=0.19, 0.66	F 1,20=4.07, 0.057	F 1,20=0.14, 0.73
		<b>PEA</b>	0.14 ± 0.02	0.13 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	F 1,20=0.17, 0.67	F 1,20=0.88, 0.18	F 1,20=0.00, 0.99
		<b>OEA</b>	0.18 ± 0.02	0.17 ± 0.01	0.19 ± 0.02	0.16 ± 0.01	F 1,20=0.03, 0.86	F 1,20=2.03, 0.16	F 1,20=0.19, 0.66
PFC	<b>AEA</b>	42.05 ± 1.54	67.69 ± 6.45	53.96 ± 8.86	53.14 ± 8.01	F 1,20=0.01, 0.89	F 1,20=3.09, 0.09	F 1,20=3.98, 0.06	
	<b>2-AG</b>	1.25 ± 0.17	1.99 ± 0.31*	1.26 ± 0.25	1.57 ± 0.19	F 1,20=0.801, 0.03	F 1,20=4.96, 0.03	F 1,20=0.38, 0.38	
	<b>PEA</b>	0.07 ± 0.00	0.11 ± 0.01*	0.12 ± 0.04	0.10 ± 0.02	F 1,19=0.21, 0.64	F 1,19=5.95, 0.02	F 1,19=0.18, 0.67	
	<b>OEA</b>	0.12 ± 0.01	0.16 ± 0.01	0.14 ± 0.03	0.14 ± 0.02	F 1,20=0.01, 0.89	F 1,20=2.43, 0.13	F 1,20=2.65, 0.11	

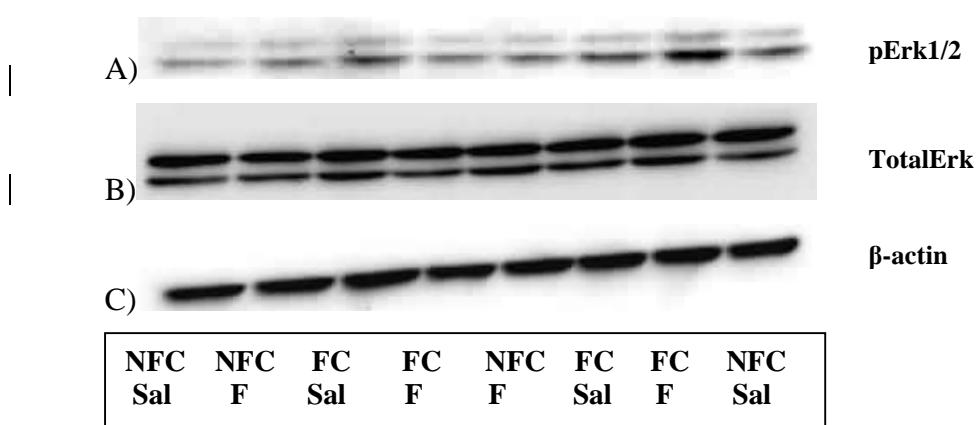
**Table 2.2 Continued**

Region	Side	Analyte	NFC-Sal	NFC-Form	FC-Sal	FC-Form	Fear conditioning (F, DF, P values)	Form (F, DF, P values)	Interaction (F, DF, P values)
BLA	left	<b>AEA</b>	3.46 ±0.73	3.67 ±0.58	7.18 ±0.32**	5.65 ±0.98	F 1,17=14.9, 0.001	F 1,17=0.79, 0.38	F 1,17=1.39, 0.25
		<b>2-AG</b>	2.68 ±1.02	4.04 ±0.55	5.32 ±0.66	6.73 ±1.93	F 1,19=4.99, 0.03	F 1,19=1.35, 0.25	F 1,19=0.00, 0.98
		<b>PEA</b>	0.05 ±0.01	0.07 ±0.01	0.12 ±0.02**	0.08 ±0.01	F 1,19=8.42, 0.009	F 1,19=0.12, 0.72	F 1,19=0.10, 0.10
		<b>OEA</b>	0.03 ±0.01	0.04 ±0.00	0.06 ±0.01**	0.05 ±0.01	F 1,19=10.12, 0.005	F 1,19=0.29, 0.59	F 1,19=3.52, 0.07
	right	<b>AEA</b>	13.21 ±1.58	16.74 ±3.06	23.82 ±2.04	15.71 ±4.13	F 1,20=2.76, 0.11	F 1,20=0.63, 0.43	F 1,20=4.09, 0.057
		<b>2-AG</b>	7.89 ±1.02	8.33 ±1.11	9.02 ±0.63	6.64 ±0.98	F 1,19=0.08, 0.76	F 1,19=1.03, 0.32	F 1,19=2.18, 0.15
		<b>PEA</b>	0.14 ±0.01	0.15 ±0.02	0.19 ±0.02	0.14 ±0.03	F 1,20=0.63, 0.43	F 1,20=1.10, 0.30	F 1,20=1.33, 0.26
		<b>OEA</b>	0.09 ±0.01	0.09 ±0.01	0.13 ±0.01*	0.08 ±0.01	F 1,20=1.14, 0.29	F 1,20=5.52, 0.03	F 1,20=5.62, 0.02
RVM		<b>AEA</b>	2.74 ±0.4	5.97 ±2.78	8.95 ±2.39*	3.55 ±1.65	F 1,20=0.87, 0.36	F 1,20=0.29, 0.53	F 1,20=4.55, 0.04
		<b>2-AG</b>	6.38 ±0.63	5.35 ±1.06	15.23 ±3.57*	7.11 ±2.16#	F 1,20=4.91, 0.06	F 1,20=3.66, 0.29	F 1,20=2.19, 0.43
		<b>PEA</b>	0.19 ±0.03	0.19 ±0.05	0.26 ±0.05	0.16 ±0.04	F 1,17=0.09, 0.76	F 1,17=0.82, 0.37	F 1,17=1.30, 0.27
		<b>OEA</b>	0.11 ±0.02	0.10 ±0.03	0.19 ±0.05	0.11 ±0.03	F 1,16=2.08, 0.16	F 1,16=1.86, 0.19	F 1,16=1.67, 0.21

\*p<0.05, \*\*p<0.01 vs. NFC-Sal, +p<0.05, ++p<0.01 vs NFC-Form, #p<0.01, ##p<0.05 vs. FC-Sal (ANOVA followed by Fisher's LSD post-hoc test); Data expressed as Mean ± SEM (n=5-6). FC, fear-conditioned; NFC, non fear-conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

### **2.3.4 Effect of fear-conditioning or/and intra-plantar formalin on levels of expression of relative pErk1/2**

In non fear-conditioned rats, intra-plantar formalin injection increased the relative expression of pErk1 by ~80% and pErk2 by ~100% in the left vIPAG, compared with saline-injected non fear-conditioned rats; however, these results failed to reach statistical significance (NFC-Sal vs. NFC-Form, **Fig. 2.6**). In contrast, there were no effects of formalin injection on pErk1/2 expression in the right vIPAG, right dlPAG, right and left BLA and RVM (NFC-Sal vs. NFC-Form, **Table 3**). Fear conditioning had no significant effect on expression of pErk1/2 in the dlPAG, vIPAG and RVM of either saline- or formalin-treated rats, compared with respective non fear-conditioned controls (NFC-Sal/Form vs. FC-Sal/Form, **Table 3**). In contrast, fear conditioning significantly increased expression of pErk1 in the right BLA of both saline- and formalin-treated rats (with a trend for a similar increase in pErk2 expression) (NFC-Sal/Form vs. FC-Sal/Form,  $p<0.05$ , **Fig. 2.9**). In rats receiving intra-plantar formalin injection, fear conditioning resulted in ~100% increase in levels of expression of relative pErk2 in the right dlPAG, though this effect did not reach statistical significance, and there was a trend for decreased levels of expression of relative pErk1/2 in the left vIPAG when compared with non fear-conditioned counterparts (NFC-Form vs. FC-Form, **Fig 2.5**).



**Figure 2.11** Representative photomicrograph of (A) pErk1/2, (B) total Erk1/2 and (C) the endogenous control  $\beta$ -actin measured in the right dlPAG following re-exposure to an observation chamber for 3 min in rats that are fear-conditioned, formalin injected or both. FC, fear-conditioned; NFC, non-fear-conditioned; Sal, Saline; F, Formalin

**Table 2.3 Relative expression of pErk1/2 in discrete brain regions of rats expressing formalin-evoked nociceptive behaviour, conditioned fear and fear-conditioned analgesia**

<b>Regions</b>		<b>NFC-Sal</b>	<b>NFC-Form</b>	<b>FC-Sal</b>	<b>FC-Form</b>	<b>Fear conditioning (F, DF, P values)</b>	<b>Form (F, DF, P values)</b>	<b>Interaction (F, DF, P values)</b>
right dlPAG	<b>pErk/TERk1</b>	107.35 ±17.7	109.45±19.37	129.79 ±26.57	144.06 ±31.67	F 1,20=1.35, 0.25	F 1,20=0.11, 0.74	F 1,20=0.06, 0.80
	<b>pErk/TERk2</b>	105.04±13.27	95.51±15.26	111.95 ±31.63	161.38 ±31.87	F 1,19=2.29, 0.14	F 1,20=0.69, 0.41	F 1,20=1.50, 0.23
left vlPAG	<b>pErk/TERk1</b>	99.46 ±12.22	173.92 ±28.69	111.70 ±38.37	123.18 ±13.92	F 1,20=0.56, 0.46	F 1,20=2.79, 0.11	F 1,20=1.50, 0.23
	<b>pErk/TERk2</b>	97.82±10.89	201.66±39.30	142.94±41.70	147.75±14.60	F 1,20=0.02, 0.88	F 1,20=3.26, 0.08	F 1,20=2.71, 0.11
right vlPAG	<b>pErk/TERk1</b>	101.14±5.23	77.38±12.37	115.64 ±35.01	107.22±29.95	F 1,19=0.77, 0.39	F 1,20=0.40, 0.053	F 1,20=0.09, 0.76
	<b>pErk/TERk2</b>	100.55 ±4.29	75.00 ±13.27	111.46±21.46	88.16±21.98	F 1,19=0.54, 0.47	F 1,20=1.35, 0.25	F 1,20=0.00, 0.96
left BLA	<b>pErk/TERk1</b>	106.14±17.00	107.58±19.49	106.65±29.27	104.28 ±33.67	F 1,20=0.00, 0.95	F 1,20=0.00, 0.98	F 1,20=0.00, 0.94
	<b>pErk/TERk2</b>	107.75 ±16.15	117.33±23.32	120.95±28.86	127.12 ±24.24	F 1,20=0.23, 0.63	F 1,20=0.11, 0.74	F 1,20=0.00, 0.94
right BLA	<b>pErk/TERk1</b>	95.75 ±11.13	88.82±19.47	183.10±33.93*	181.42±38.61+	F 1,20=10.3, 0.0004	F 1,20=0.02, 0.88	F 1,20=0.00, 0.92
	<b>pErk/TERk2</b>	102.33 ±15.53	106.21±22.87	146.22 ±28.80	135.77±16.20	F 1,20=2.9, 0.10	F 1,20=0.02, 0.88	F 1,20=0.11, 0.74
RVM	<b>pErk/TERk1</b>	100.93±10.84	110.98±26.27	133.34±12.90	136.58±7.35	F 1,20=3.81, 0.06	F 1,20=0.20, 0.65	F 1,20=0.05, 0.82
	<b>pErk/TERk2</b>	101.95 ±5.79	120.94 ±2.72	137.70±13.56	107.44±8.83	F 1,20=0.25, 0.61	F 1,20=0.06, 0.80	F 1,20=1.25, 0.27

\*p<0.05 vs. NFC-Sal, +p<0.05 vs. NFC-Form (ANOVA followed by Fisher's LSD posthoc test). Data expressed as % NFC-Sal ± SEM (n=5-6). FC, fear-conditioned; NFC, non fear-conditioned; Sal, Saline; Form, Formalin.

## **2.4 Discussion**

The present experiment demonstrated differential response of endocannabinoids and related lipids during conditioned fear or following a noxious inflammatory stimulus (formalin) in rats depending on the brain regions investigated. The expression of FCA (i.e. conditioned fear-induced suppression of formalin-evoked nociceptive behaviour) was also associated with alterations in levels of endocannabinoids and NAEs in discrete brain regions. In addition, formalin-induced nociceptive behaviour was associated with a strong trend towards increased expression of Erk1/2 in the vIPAG; whereas, both expression of conditioned fear and FCA were accompanied by increased expression of Erk1 in the right BLA. Together, these data constitute novel and specific neurochemical and molecular correlates of conditioned fear, inflammatory pain and FCA in rats.

Fear conditioning resulted in significant expression of fear behaviours during re-exposure to the conditioned-stimulus (i.e. the context). To further investigate the potential neurochemical mechanisms underpinning the behavioural effects observed, we measured tissue concentrations of the endocannabinoids, AEA and 2-AG, and the related ‘entourage’ NAEs, PEA and OEA, in discrete brain regions of rats sacrificed 3min following re-exposure to context, a time-point where maximal expression of fear-related behaviour and FCA was noted (see **Fig 3.3B** of Chapter 3). The PAG and BLA are key brain regions involved in coordinating the defence response to aversive stimuli (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux *et al.*, 1988b; Schenberg *et al.*, 1990; Vianna *et al.*, 2003) and endocannabinoid signalling in these brain regions plays a vital role in the modulation of behavioural responses to unconditioned (Bortolato *et al.*, 2006; Connell *et al.*, 2006; Kathuria *et al.*, 2003; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Patel *et al.*, 2006a; Patel *et al.*, 2009) and conditioned (Broiz *et al.*, 2008; Chhatwal *et al.*, 2007; Fendt *et al.*, 1996; Finn *et al.*, 2004; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Marsicano *et al.*, 2002; Resstel *et al.*, 2008; Tan *et al.*, 2011) stress. We report a fear-related increase in levels of all analytes in the right dlPAG, increased PEA in the left dlPAG, increased 2-AG in the vIPAG, increased AEA, OEA and PEA in the left BLA

and increased OEA in the right BLA 3min following re-exposure to conditioned stressor. Previously, Hohmann and colleagues have shown increased levels of 2-AG and AEA in the rat dlPAG at 2-7min and 7-25min respectively, following footshock (Hohmann *et al.*, 2005). Moreover, re-exposure to a tone paired previously with footshock resulted in increased AEA and 2-AG levels in the basolateral amygdala of mice 3min following re-exposure (Marsicano *et al.*, 2002). In addition, 2-AG was increased in amygdala and prefrontal cortex after chronic restraint stress in mice (Rademacher *et al.*, 2008). Our data support and extend these findings by demonstrating that Pavlovian conditioned fear to a context mobilises endocannabinoids in the dlPAG, vlPAG and BLA. Fear-related increases in the non-endocannabinoid NAEs, OEA and PEA, were also observed in the dlPAG and BLA. Although Hill *et al.* (2009) demonstrated that peripheral NAEs are responsive to stress, to our knowledge, the present results represent the first report on the effects of conditioned fear on levels of these ‘entourage’ compounds in the brain. Although these fatty acid amides are devoid of significant activity at the CB<sub>1</sub> receptor (Appendino *et al.*, 2006), by competing as substrates for FAAH, OEA and PEA may in turn enhance the actions of AEA at CB<sub>1</sub> by limiting its degradation. Similarly, in the RVM, which is known for its role particularly in the emotional motor system (Vianna *et al.*, 2008), conditioned fear was accompanied by increased levels of AEA and 2-AG. It appears that upon exposure to stressful stimuli, central endocannabinoids are mobilised in key brain areas such as PAG, amygdala and RVM, which might contribute to adaptive and emotional strategies to cope with aversive situations. It seems reasonable to speculate that the fear-related increases in one or more of these lipids in these brain regions may play a key role in mediating conditioned fear behaviours as well as FCA.

Despite the well-established role of the endocannabinoids in the hippocampus (Campos *et al.*, 2010; de Oliveira Alvares *et al.*, 2010; Llorente *et al.*, 2008; Rubino *et al.*, 2008a; Viveros *et al.*, 2007) and cortex (Laviolette *et al.*, 2006; Lin *et al.*, 2009; Rademacher *et al.*, 2008; Rubino *et al.*, 2008a; Rubino *et al.*, 2008b) in fear/anxiety-related behaviour, levels of analytes in these brain regions were largely unaffected by conditioned fear in the present study,

although increased levels of PEA associated with conditioned fear were observed in the left dorsal hippocampus. Bilateral administration of URB597 into the ventral hippocampus enhanced FCA in a CB<sub>1</sub>-dependent manner suggesting an important role for the endocannabinoid system in the ventral hippocampus in FCA (Ford *et al.*, 2011). It is possible endocannabinoids in these brain regions may have been altered at a different time point following re-exposure to conditioned-stressor or perhaps are released in these regions in response to different forms of stress.

Intra-dlPAG levels of 2-AG, OEA and PEA were significantly increased in saline-injected rats following fear conditioning, whereas, in formalin-treated rats, levels of these same analytes were not significantly altered by fear conditioning (though some trends towards an increase were seen). In addition, fear-induced alterations in levels of endocannabinoids and/or NAEs observed in the vIPAG, BLA and RVM of saline-injected rats were not seen in that of formalin-injected rats. These data suggest differential effects of conditioned fear on levels of 2-AG, OEA and PEA in the presence versus absence of nociceptive tone. The mechanism(s) responsible for such a state-dependent alteration in the responsivity of these analytes to conditioned fear are unknown, however it does not appear to be due to formalin-evoked alterations in absolute levels of the analytes since there were no significant effects of intra-plantar formalin injection on tissue concentrations of any of the 4 analytes in these regions under the present experimental conditions. As discussed in Chapter 1 however, there is very significant overlap in the neural substrates, brain regions and circuitry involved in pain and fear/anxiety. It is possible therefore that pain-induced alterations in neuronal activity, neurotransmission and/or neurochemistry within these regions could in turn influence fear-related mobilisation of endocannabinoids and NAEs in the same regions.

Intra-plantar injection of formalin decreased levels of AEA in the left IPAG and 2-AG in the right IPAG without affecting levels in other sub-regions of the PAG or in the BLA or RVM. These results differ from previous studies which have reported pain-related increases in tissue levels of endocannabinoids in discrete

brain regions including the PAG and RVM. For example, in rats, mechanical allodynia and thermal hyperalgesia following spinal nerve ligation were accompanied by increased levels of AEA and 2-AG in the PAG, RVM, dorsal raphe magnus and dorsal root ganglia (Mitrirattanakul *et al.*, 2006; Petrosino *et al.*, 2007). Using an approach employing *in vivo* microdialysis, Walker *et al.* (1999) demonstrated increased levels of extracellular AEA in the rat dorsal and lateral PAG following formalin injection. However, direct comparisons between these earlier studies and the present study are difficult to make due to differences in the models used (spinal nerve ligation versus formalin test), dose of the formalin administered (4%, 150 $\mu$ l into both hind paws *vs.* 2.5%, 50 $\mu$ l into right hind paw), time-points and sub-regions assayed and method of analysis (microdialysis *vs.* tissue levels). The decrease in levels of endocannabinoids in lPAG following noxious stimuli is intriguing and could mean decreased production or increased destruction. However, as endocannabinoids are produced ‘on demand’ increased destruction could be the most likely mechanism. In contrast to our finding in the PAG and BLA, we found that intra-plantar formalin increased levels of AEA in the left insular cortex and 2-AG and PEA in the mPFC. These alterations could represent adaptive reactions aimed at reducing pain and could occur in other brain regions at a different time point. It is well established that ascending nociceptive information is relayed contralaterally to the brain (Ghosh *et al.*, 2009; Kalliomaiki *et al.*, 1993; Schouenborg *et al.*, 1986). As formalin was injected only into the right hind paw in the present study, the lateralisation observed here in endocannabinoid response in the left versus right insula could be attributed to the fact that the contralateral brain region activated by the ascending pathway responds differently to the ipsilateral (right) side. While the changes described above were in non fear-conditioned rats, following fear conditioning, intra-plantar formalin decreased levels of 2-AG in left dlPAG, vlPAG, and RVM. These data suggest differential effects of conditioned fear on levels of 2-AG in the presence versus absence of nociceptive tone.

The suppression of formalin-evoked nociceptive behaviour observed here upon re-exposure, for 3min, to a context previously paired with footshock is similar in

its nature and magnitude to previous reports demonstrating FCA using related or identical paradigms with longer duration of exposure (Butler *et al.*, 2008; Finn *et al.*, 2004; Helmstetter *et al.*, 1987; Roche *et al.*, 2007). The expression of FCA was associated with increased levels of AEA in the left lPAG and right dlPAG but decreased levels of 2-AG in the left dlPAG. Previously, Hohmann *et al.*, reported that increased endocannabinoid levels in the dlPAG, following exposure to stress, mediate SIA through activation of the descending inhibitory pathway that propagates to the dorsal horn of the spinal cord (Hohmann *et al.*, 2005). It is possible that increased AEA in the dlPAG and lPAG also mediates the suppression of pain by conditioned psychological stress (i.e. FCA). Future work involving pharmacological blockade of CB<sub>1</sub> receptors in these brain regions may confirm the role of the endocannabinoid system in these brain regions in FCA (e.g. see Chapter 3). The differences in both the direction of change and the type of analyte involved in the left and right dlPAG is intriguing. However, it has been reported that changes of endocannabinoid in the same direction often accompany disorders with opposing symptoms; or levels of AEA and 2-AG change in different or even opposing ways during the same condition (Di Marzo *et al.*, 2007; Pacher *et al.*, 2006). In addition, such lateralisation of endocannabinoid response could be due to the fact that formalin was administered to the right hind paw and the contralateral hemisphere was activated differentially.

MAPK has been shown to be activated in the spinal cord, and supraspinal brain regions such as hypothalamus, amygdala and PAG following noxious stimulation (Carrasquillo *et al.*, 2008; Carrasquillo *et al.*, 2007; Choi *et al.*, 2006; Gioia *et al.*, 2005; Karim *et al.*, 2001). Here, intra-plantar formalin increased the relative expression of pErk1/2 in the left vIPAG (though this result failed to reach statistical significance) without affecting levels in other PAG sub-regions. However, these changes were not accompanied by alterations in endocannabinoid levels at the same time point. The PAG is involved in both the ascending pain system (Keay *et al.*, 1997) and in descending pain inhibition (Bandler *et al.*, 1994; Behbehani, 1995; Westlund, 2000). Previously, visceral noxious stimulation was shown to activate Erk1/2 levels in the intermediate and

caudal neurons of the IPAG, vIPAG, and dPAG columns; whereas, neurons of the most rostral PAG did not seem to be involved (Gioia *et al.*, 2005). However, direct comparison is not possible as the discrepancy could be due to the variation in the methodology, primarily the time of assessment (33min vs. 2hr), the pain model (intra-plantar formalin vs. intra-peritoneal acetic acid) and the method used to measure Erk1/2 levels (western blotting vs. immunohistochemistry). The activation of Erk which was limited to the left vIPAG here could be due to the unilateral (right side) injection of formalin. However, it has been reported that irrespective of whether inflammation was induced in the right or left paw Erk activation could be observed only in one side of the brain as was the case in the right central nucleus of the amygdala (Carrasquillo *et al.*, 2008). The present study found no significant effects of formalin injection on pErk1/2 expression in the right and left BLA or in the RVM. This is in contrast to previous work which showed Erk1/2 activation following noxious stimuli in the amygdala (Carrasquillo *et al.*, 2007) and RVM (Imbe *et al.*, 2008; Imbe *et al.*, 2004; Imbe *et al.*, 2005). Methodological differences could be the reason for these discrepancies.

Fear conditioning significantly increased expression of pErk1 in the right BLA of both saline- and formalin-treated rats (with a trend for a similar increase in pErk2 expression). This is in line with previous reports that amygdalar MAPK activation is important in the acquisition and consolidation of conditioned fear (Di Benedetto *et al.*, 2008; Duvarci *et al.*, 2005; Schafe *et al.*, 2000). The fear-induced increase in pErk1 was accompanied by increased levels of OEA and strong trend for increased AEA in the right BLA. It is possible that increased AEA during conditioned-fear may activate CB<sub>1</sub> receptors to result in increased level of pErk1 in the BLA. Supporting this notion, the expression of conditioned fear is associated with increased pErk expression in the BLA, an effect which was CB<sub>1</sub> receptor-dependent (Cannich *et al.*, 2004). The fear-related increase in OEA may in turn potentiate the effects of AEA via the ‘entourage’ effect. OEA is also shown to activate Erk pathway in cardiac muscle(Su *et al.*, 2006) although such effect on neurons has not been shown. In addition the fact that FCA was associated with increased pErk1 is in the right BLA in line with

previous work showing an increase in pErk1/2 in the amygdala (Butler *et al.*, 2008) and suggests a possible role for Erk1 in the BLA in the mediation of FCA. The reason for fear-induced alteration in pErk1 but not in pErk2 is not clear; however, a similar differential alteration of pErk1 and pErk2 was seen previously in the BLA during conditioned-fear (Cannich *et al.*, 2004). The fact that pErk expression was only altered in the right BLA is in line with lateralisation demonstrated with the right amygdala showing greater involvement in fear conditioning than the left (Baker *et al.*, 2004). In addition, neuroanatomical (Adolphs *et al.*, 1996), electroencephalograph (Ahern *et al.*, 1985), and brain imaging studies (Canli *et al.*, 1998) also suggest hemispheric lateralization (i.e., right hemispheric dominance) in aversive emotional processing and expression.

FCA which was associated with increased levels of AEA in the right dlPAG, was also accompanied by a strong trend towards increased levels of pErk1/2 in the right dlPAG. This suggests a possibility that AEA-induced activation of pErk in the dlPAG following fear conditioning might mediate FCA. However, it should be noted that the results we obtained here did not achieve statistical significance. Despite the fear-induced increase of endocannabinoids in the vIPAG and RVM, these changes were not accompanied by alteration in expression of pErk under the present experimental condition. Previously, using this same model of endocannabinoid-mediated FCA, pErk1/2 expression was found to be unchanged in the PAG, hippocampus, prefrontal cortex, and thalamus (Butler *et al.*, 2008) and was decreased in the PFC (Butler *et al.*, 2011) following FCA. Butler *et al.* (2008) also argued against a role for Erk1/2 signalling in the amygdala during expression of FCA.

In conclusion, the present data constitute a detailed characterisation of endocannabinoid and NAE levels in brain regions that are involved in fear, nociception and FCA. It is possible that alterations in levels of endocannabinoids and/or NAEs in these brain regions might mediate, at least in part, fear-induced modulation of nociception. Furthermore, the results suggest the possibility that pErk1/2 expression in the dlPAG and BLA may be involved

***Chapter 2: Characterisation of brain regional levels of endocannabinoids and N-acylethanolamines during expression of conditioned fear, pain or fear-conditioned analgesia in rats***

in the expression of endocannabinoid-mediated FCA. These data provide a solid foundation upon which to design further mechanistic studies aimed at elucidating the neural substrates and neurochemical and molecular mechanisms underpinning endocannabinoid-mediated FCA.

***Chapter 3: The role of the endocannabinoid system in the rat dorsolateral periaqueductal grey in pain, conditioned fear and fear-conditioned analgesia***

### ***3.1 Introduction***

The periaqueductal grey (PAG) is a mesencephalic structure that can be divided into four columns along its rostro-caudal axis: the dorsomedial, dorsolateral, lateral and ventrolateral columns (Bandler *et al.*, 1996). It is a key component both of the circuitry responsible for anxiety-related defence responses (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux, 1998; Schenberg *et al.*, 1990) and of the descending inhibitory pain pathway (Helmstetter *et al.*, 1998; Millan, 2002; Oliveira *et al.*, 2001; Pavlovic *et al.*, 1998). The dl part of PAG is important in the descending inhibitory control of pain (Haghparast *et al.*, 2009; McMullan *et al.*, 2006; Waters *et al.*, 2008) and modulation of aversive responses (Bertoglio *et al.*, 2005; Brandão *et al.*, 1999; Canteras *et al.*, 1999; Fontani *et al.*, 1983; Klein *et al.*, 2010; Lino-de-Oliveira *et al.*, 2006; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Resstel *et al.*, 2008). The PAG is also known to play a key role in mediating analgesia induced by stress or fear (Butler *et al.*, 2011b; Helmstetter *et al.*, 1990; Hohmann *et al.*, 2005). Furthermore, lesions of the dlPAG (Helmstetter *et al.*, 1994; Kinscheck *et al.*, 1984) have been shown to reduce or abolish the expression of FCA in rats, and stimulation of the dorsal PAG induces FCA which is attenuated by enhancing GABAergic transmission in this region (Castilho *et al.*, 2002).

As highlighted in earlier sections, several studies have indicated a role for the endocannabinoid system in the suppression of pain responding during or following exposure to either unconditioned or conditioned stress. For example, previous work has shown that FCA is prevented by systemic administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, (Finn *et al.*, 2004) and enhanced by systemic administration of the FAAH inhibitor URB597 (Butler *et al.*, 2008; Butler *et al.*, 2011a). Hohmann and colleagues have demonstrated an important role for the endocannabinoid system in the dlPAG in mediating unconditioned SIA expressed as a suppression of tail-flick responding following exposure of rats to unconditioned footshock stress (Hohmann *et al.*, 2005).

Specifically, this form of analgesia evoked by unconditioned physical stress was blocked by intra-dlPAG administration of the CB<sub>1</sub> receptor antagonist/inverse agonist rimonabant and enhanced by intra-dlPAG administration of the FAAH inhibitors, arachidonoyl serotonin (AA-5-HT) and URB597, or the MAGL inhibitor, URB602 (Hohmann *et al.*, 2005; Suplita *et al.*, 2005). The PAG contains a moderate density of CB<sub>1</sub> receptors (Herkenham *et al.*, 1991; Tsou *et al.*, 1997) and it has been shown that the expression of CB<sub>1</sub> receptors in the CNS exhibits a similar distribution pattern to FAAH (Egertová *et al.*, 2003; Thomas *et al.*, 1997), and MAGL (Tsuyama *et al.*, 2007). However, the role of the endocannabinoid system in the PAG in analgesia induced by conditioned psychological stress/fear (FCA) has not been examined. In addition, no studies to date have investigated the role of the endocannabinoid system in the PAG in fear expressed in the presence of nociceptive tone.

Activation of both the CB<sub>1</sub> and CB<sub>2</sub> receptors leads to the activation of the MAPK pathway through phosphorylation of Erk1/2 (Bouaboula *et al.*, 1995a; Bouaboula *et al.*, 1996). Furthermore, MAPK activation in the CNS has been reported following noxious stimulation (Butler *et al.*, 2011b; Carrasquillo *et al.*, 2008; Carrasquillo *et al.*, 2007; Choi *et al.*, 2006). Amygdalar MAPK activation is important in the acquisition and consolidation of conditioned fear (Di Benedetto *et al.*, 2008; Duvarci *et al.*, 2005; Schafe *et al.*, 2000) and expression of FCA is associated with increased pErk2 in the amygdala, an effect attenuated by the CB<sub>1</sub> receptor antagonist/agonist, rimonabant (Butler *et al.*, 2008). In addition, a formalin-evoked increase in expression of pErk1/2 in the PFC was attenuated following fear conditioning (Butler *et al.*, 2011b). However, pErk1/2 expression was unchanged in the PAG, hippocampus, prefrontal cortex, and thalamus following FCA (Butler *et al.*, 2008), suggesting region-specific effects. Systemic administration of URB597 significantly increased levels of relative pErk1/2 expression in the PAG of rats expressing FCA without affecting the relative pErk1/2 expression in non-fear-conditioned rats. It was shown in chapter 2 that the expression of FCA was not associated with alterations in the expression level of pErk1/2 in the dl and vlPAG though strong trends towards increased and decreased expressions were observed, respectively. However, the

exact role of endocannabinoid-mediated modulation of pErk1/2 activity in the PAG during expression of pain, fear and FCA is still poorly understood.

Monoamines (NA, 5-HT, DA), in the PAG and RVM have been extensively implicated in the modulation of fear/anxiety state and descending control of pain (for review see (Millan, 2002; Millan, 2003). Several studies have demonstrated that cannabinoid receptor activation can modulate (Bambico *et al.*, 2007; Kathmann *et al.*, 1999; Mendiguren *et al.*, 2006; Oropeza *et al.*, 2005) or be modulated by (Carta *et al.*, 1999; Lichtman *et al.*, 1991) monoaminergic neurotransmitter systems. For example, cannabinoids produce antinociception, in part, by modulating descending noradrenergic systems (Gutierrez *et al.*, 2003). Evidence also suggests involvement of brain monoaminergic systems in endocannabinoid-mediated modulation of behavioural despair or stress coping (Gobbi *et al.*, 2005; Takahashi *et al.*, 2008). Subthreshold doses of rimonabant or AM251 were effective in the forced swim or tail suspension tests when co-administered with selective serotonin re-uptake inhibitors (Takahashi *et al.* 2008). In addition, systemic administration of the FAAH inhibitor URB597 exerted potent antidepressant-like effects in the mouse forced swim and tail suspension tests together with increased firing of serotonergic neurons in the dorsal raphe' nucleus and noradrenergic neurons in the locus coeruleus (Gobbi *et al.*, 2005) Furthermore, several studies to date have reported the role of the serotonergic (Kavaliers *et al.*, 1997b; Nunes-de-Souza *et al.*, 2000; Watkins *et al.*, 1984), dopaminergic (Bambico *et al.*, 2007; Snow *et al.*, 1982) and noradrenergic (Bodnar *et al.*, 1983; Snow *et al.*, 1982; Watkins *et al.*, 1984) systems in SIA/FCA. In addition FCA is associated with alterations in dopaminergic and serotonergic systems in the cerebellum (Roche *et al.*, 2007) and alterations in dopaminergic transmission in the PAG and thalamus (Finn *et al.*, 2006). However, our understanding of endocannabinoid–monoaminergic interactions in the expression of conditioned fear, nociception and FCA is still poor. PAG neurons projecting to serotonergic and noradrenergic neurons in the RVM and pontine nuclei are believed to exert inhibitory effects on spinal nociceptive functions through the activation of descending serotonergic and noradrenergic pathways (Odeh *et al.*, 2001). In addition, Holstege *et al.*, (1996) described “the emotional motor system” where the RVM plays an important role

by relaying outputs from the hypothalamus and PAG during fear (Vianna *et al.*, 2008). In chapter 2, we have shown that conditioned fear is accompanied by increased levels of AEA and 2-AG in the RVM. The RVM is therefore an important integrating centre in both the descending pain pathway and the descending emotional motor system. However, the extent to which pharmacological or environmental (aversion or/and nociception) modulation of the endocannabinoid system in the PAG alters monoamine levels in the RVM is not known.

In addition to the modulation of nociceptive responding, the endocannabinoid system in the PAG is also a critical site for the regulation of fear responding. An increased understanding of the role of the endocannabinoid system in conditioned fear is important because a number of anxiety disorders such as post traumatic stress disorder and phobias are thought to result from a persistent maladaptive expression of this important defence behaviour. Evidence suggests that endocannabinoid signalling in the PAG plays an important role in the modulation of behavioural responses to unconditioned (Bortolato *et al.*, 2006; Kathuria *et al.*, 2003; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Patel *et al.*, 2006a) and conditioned (Broiz *et al.*, 2008; Chhatwal *et al.*, 2007; Fendt *et al.*, 1996; Finn *et al.*, 2004; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Marsicano *et al.*, 2002; Ressell *et al.*, 2008) stress. Differential modulation of anxiety at different doses of URB597 has been demonstrated following both systemic (Scherma *et al.* 2007) and intra-cerebral administration (Rubino *et al.*, 2008b), with low doses inducing anxiolysis and high doses inducing anxiety or having no effect. However it is unknown if enhancing endocannabinoid tone within the PAG following intra-PAG injection of URB597 would elicit similar differential effects on conditioned fear-related behaviour.

The objectives of the studies described in this chapter were:

- To determine the role of the endocannabinoid system in the dlPAG in formalin-evoked nociceptive behaviour, expression of FCA, and expression of fear behaviour in the presence of formalin-evoked nociceptive tone. This was achieved by examining the effects of intra-dlPAG administration of the FAAH

inhibitor, URB597, or the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, on formalin-induced nociceptive behaviour, fear-related behaviour in the presence of nociceptive tone and FCA in rats

- To examine if behavioural changes are associated with alterations in levels of pErk1/2 in the dlPAG and monoamine levels in the RVM.
- A further aim of the studies was to determine if conditioned fear behaviour was altered by varying the dose of URB597 administered into the dlPAG and examining the associated alterations in monoamine levels in the RVM.

The work tests the hypothesis that enhanced endocannabinoid activity in the dlPAG mediates antinociception, anxiolysis and FCA.

### **3.2 Methodology**

#### **3.2.1 Animals**

Experiments were carried out on adult male Lister-Hooded rats (240–310g at the time of experimentation; Charles River, Kent, UK) maintained at a constant temperature ( $21 \pm 2^{\circ}\text{C}$ ) under standard lighting conditions (12:12h light: dark, lights on from 07.00 to 19.00h). All experiments were carried out during the light phase between 08.00h and 17.00h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

#### **3.2.2 Drug preparation**

The CB<sub>1</sub> receptor antagonist/inverse agonist rimonabant (SR141716A; (N-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1-H-pyrazole-3-carboxamide], NIMH Chemical Synthesis Programme Batch 10937-163-1) and the FAAH inhibitor URB597 [(30-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) Cayman Chemical Company, Tallinn, Estonia] were prepared on day of use to a concentration of 0.4nmol / 0.2μl (2mM) (rimonabant) and 0.02nmol/0.2μl (0.1mM), 0.1nmole/0.2μl(0.5mM) and 0.5nmole/0.2μl(2.5mM) (URB597) in DMSO (dimethylsulfoxide, 100%). These concentrations of rimonabant and URB597 were chosen based on pilot studies in our laboratory and previous work demonstrating attenuation and enhancement, respectively, of unconditioned SIA in rats when injected into the dorsal PAG (Hohmann *et al.*, 2005; Suplita *et al.*, 2005).

#### **3.2.3 Cannula implantation**

Under isoflurane (2-3% in O<sub>2</sub>, 0.5L/min) anaesthesia, a stainless steel guide cannula (9mm length, Plastics One Inc., Roanoke, Virginia, USA) was stereotactically implanted 1mm above the right dlPAG of each rat (coordinates: AP = - 6.3mm from bregma, ML = + 1.9mm at an angle of 16°, DV = 4.0mm from the meningeal dura matter according to the rat brain atlas published by Paxinos and Watson, 1997 (Paxinos *et al.*, 1997). The cannulae were permanently fixed to the skull using stainless steel screws and carboxylate

cement. A stylet made from stainless steel tubing (18 mm, 31 G) (Plastics One Inc., Roanoke, Virginia, USA) was inserted into the guide cannula to prevent blockage by debris. The non-steroidal anti-inflammatory agent, carprofen (1.25mg/25 $\mu$ L, s.c., Rimadyl, Pfizer, Kent, UK), was administered before the surgery to manage postoperative analgesia. Animals received a single daily dose of the antimicrobial agent enrofloxacin (10mg/kg, s.c., Batyril, Bayer plc, Berkshire, UK) for 5 days to prevent postoperative infection. Following cannula implantation, the rats were housed singly and at least 6 days were allowed for recovery post-surgery prior to experimentation. During this recovery period, the rats were handled, stylets checked, and their body weight and general health monitored once daily.

### **3.2.4 Experimental procedure**

FCA was modelled as described previously in Chapter 2 (Section 2.2.2).

**3.2.4.1 Experiment 1:** The test phase commenced 23.5h later when the subjects received an intraplantar injection of 50 $\mu$ L formalin (2.5% in 0.9% saline) into the right hind-paw under brief isoflurane anaesthesia (3% in O<sub>2</sub>; 0.5L/min). Rats were returned to their home cage for a further 15min, after which time they received a single intra-dlPAG microinjection (0.2 $\mu$ L) of URB597 (0.02nmol), rimonabant (0.4nmol) or vehicle (100% dimethyl sulfoxide; DMSO) as described previously (Finn et al., 2003, Roche et al., 2007).

Drugs were microinjected manually into the right dlPAG in a volume of 0.2  $\mu$ L using an injector and Hamilton syringe attached to 50-cm-long polyethylene tubing (0.75 mm outside diameter, 0.28 mm inside diameter, Harvard Apparatus, Kent, UK) to minimise handling and enable injections to be carried out while the rats remained in the home cage. Drugs were microinjected over a period of 1min and the needle was left in position for 1min to allow diffusion of the drug before the cannula was withdrawn.

This design resulted in six experimental groups: fear-conditioned + vehicle (FC-Veh); fear-conditioned + rimonabant (FC-Rim); fear-conditioned + URB597 (FC-URB); non fear-conditioned + vehicle (NFC-Veh); non fear-conditioned +

Rimonabant (NFC–Rim); non fear-conditioned + URB597 (NFC–URB). Following intra-dlPAG injection, rats were returned to their home cage until 30min post-formalin injection after which time they were placed back in the perspex observation chamber to which they had been exposed during the conditioning phase. A bat detector (Batbox Duet, Batbox, Steyning, West Sussex, and UK) was used to detect ultrasonic vocalization in the 22 kHz range, and behaviours were recorded for 15min with the aid of a video camera located beneath the observation chamber. The 30–45min post-formalin interval was chosen on the basis of previous studies demonstrating robust suppression of formalin-evoked nociceptive behaviour upon re-exposure to an aversively conditioned context during this part of the second phase formalin response (Finn *et al.*, 2004; Finn *et al.*, 2006; Rea *et al.*, 2009a; Roche *et al.*, 2009; Roche *et al.*, 2007), and previous work demonstrating that such FCA expressed during this period is CB<sub>1</sub> receptor-mediated (Butler *et al.*, 2008; Finn *et al.*, 2004).

Rats were decapitated at the end of the test trial and 0.2µL 2% fast-green dye (dissolved in DMSO) was microinjected via the guide cannula to mark the site of injection. Following removal of the brain, a block of tissue containing the injection site (PAG) was removed, snap-frozen on dry ice and stored at -80°C for subsequent histological verification of cannula positioning in the right dlPAG and tissue collection of the right dlPAG using Palkovits punch method for western immunoblotting. The RVM was dissected out on an ice cold plate and stored at -80°C until processed for monoamine determination using HPLC.

**3.2.4.2 Experiment 2:** A separate cohort of rats underwent a similar experimental protocol to that described above but without intra-plantar injection of formalin. This design comprised a conditioning phase on day 1 followed 24hrs later by intra-dlPAG administration of one of several doses of URB597 (0.1, 0.5, 2.5mM) or vehicle (100% DMSO) using an injector and Hamilton syringe as described above. Rats were returned to their home cage until 10min post-intra-PAG injection, after which they were placed back in the Perspex observation chamber to which they had been exposed during the conditioning phase. This design resulted in five experimental groups: no-fear-conditioning + Vehicle (NFC-Veh); fear-conditioning + Vehicle (FC-Veh); fear-conditioning +

0.1mM URB597 (FC-0.1); and fear-conditioning + 0.5mM URB597 (FC-0.5), fear-conditioning + 2.5mM URB597 (FC-2.5mM). A bat detector was used to detect ultrasonic vocalization in the 22kHz range, and behaviours were recorded for 10min with the aid of a video camera as described above. The 10min post-drug injection time was chosen on the basis of previous studies where URB597 administered to dlPAG at this time point induced anxiety-like behaviour (Lisboa *et al.*, 2008). At the end of the experiment animals were decapitated, and 0.2 $\mu$ L 2% fast-green dye injected into the dlPAG prior to brain removal and subsequent histological verification of injector site placement as described above. The RVM was dissected out on an ice cold plate and stored at -80°C until processed for monoamine determination using HPLC.

### **3.2.5 Cryosectioning of the dlPAG for identification of injection site and tissue isolation by Palkovits punch method**

The block of tissue containing the PAG was sectioned (30 $\mu$ m) on a cryostat (MICROM GMBH, Germany), and sections containing the region of interest, identified by the presence of fast green dye, were mounted on glass slides for counter-staining with Cresyl Violet. Prior to and following the collection of sections to confirm cannula placement, sections of 300 $\mu$ m thickness, were taken and the right dlPAG was isolated from the frozen sections using cylindrical brain punches (Harvard Apparatus, internal diameter 0.75mm). A maximum of six 300 $\mu$ m thick sections were used for this purpose (Bregma, -5.8→ -8.0mm). Samples were immediately frozen on dry ice and stored at -80°C until processed for western immunoblotting.

### **3.2.6 Histology**

The site of injection was determined prior to data analysis. Cryosections containing the right dlPAG mounted on glass slides were dehydrated in graded alcohols as follows: briefly dipped in distilled water followed by 5min in 0.1% Cresyl Violet (Sigma Aldrich Ireland, Ltd., Dublin, Ireland), 1 min in 50% ethanol, 1 min 70% ethanol, 2 min in 100% ethanol, 2 min in Xylene, and then 5 min in xylene. Drops of DPX mountant for microscopy (VWR International Ltd., Poole, England) were then put onto the slides after which the slides and

stained sections were covered with a glass coverslip. The precise position of the injector tips were confirmed under a light microscope.

### **3.2.7 Behavioural analysis**

Behaviour was analysed as described in Chapter 2 (Section 2.2.3).

### **3.2.7 Western immunoblotting**

dIPAG tissues which were isolated using Palkovits punch method described above were processed for western immunoblotting essentially as described in Chapter 2 (Section 2.2.6). The only change to this protocol was the constitution of some of the buffers: washing buffer (0.5% Tween 20 in TBS), blocking solution (5% milk, 0.5% Tween 20 in TBS), primary antibody diluent (2.5% milk, 0.05% Tween 20 in TBS) and secondary antibody diluent (2.5% BSA, 0.05% Tween 20 in TBS). Secondary antibody dilution of 1:10,000 was used in this present protocol. The Bradford protein assay was performed as described in Chapter 2 (Section 2.2.7).

### **3.2.8 High-pressure liquid chromatography (HPLC) analysis of brain tissue monoamine concentrations**

RVM tissues (average weight-  $5.63 \pm 0.34\text{mg}$ ) were thawed and sonicated in 1ml of mobile phase (0.1M citric acid, 0.1M NaH<sub>2</sub>PO<sub>4</sub>, 1.4mM 1-octane sulphonic acid, 0.01mM EDTA, 10% methanol; pH 2.8) containing 2ng/20.0 $\mu\text{L}$  N-methyl 5-HT as an internal standard, and homogenates were centrifuged at 4°C for 15min at 14,000g. The HPLC method used was based on that of Seyfried et al. (1986) and is essentially as described by Roche et al., 2007. A 20 $\mu\text{l}$  sample of supernatant was injected onto a Shimadzu HPLC with a reverse-phase C18 column (Licosorb RP-18 column; Phenomenex, Macclesfield, Cheshire, UK). Electrochemical detection was used to determine peak heights of monoamines and their metabolites. The electrode was maintained at +0.8V and the flow rate of the mobile phase through the system was 1 ml/min. Peak heights (PH) for a mixture of standards (Mix) comprised of 2ng/20 $\mu\text{l}$  of 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5- HIAA) and the internal standard N-methyl-5-HT(IS) (Sigma-Aldrich Ireland, Dublin, Ireland)

were obtained each day prior to injection of samples, and after every 10 samples. The data were expressed as ng neurotransmitter/g of tissue.

This involved 3 steps:

1. Determination of relative retention factor (RRF):

The RRF describes the ability of an HPLC system to discriminate between two compounds i.e. a reference compound (IS) and an unknown compound (NT in the tissue). RRF was determined using the following formula:

$$\text{RRF} = (\text{Conc IS}_{\text{Mix}} \times \text{PH NT}_{\text{Mix}}) \div (\text{Conc NT}_{\text{Mix}} \times \text{PH IS}_{\text{Mix}})$$

2. Determination of the concentration of a particular NT in the brain tissue sample as ng NT per 20 $\mu$ l:

$$[\text{NT}] (\text{ng}/20\mu\text{l}) = (\text{PH NT} \times \text{Conc IS}) \div (\text{PH IS} \times \text{RRF NT})$$

3. To convert NT concentration from units of ng/20 $\mu$ l to units of ng/gram tissue

$$[\text{NT}] (\text{ng}/\text{gram tissue}) = [([\text{NT}] \text{ sample} \times 50) \div \text{weight sample (mg)}] \times 1000$$

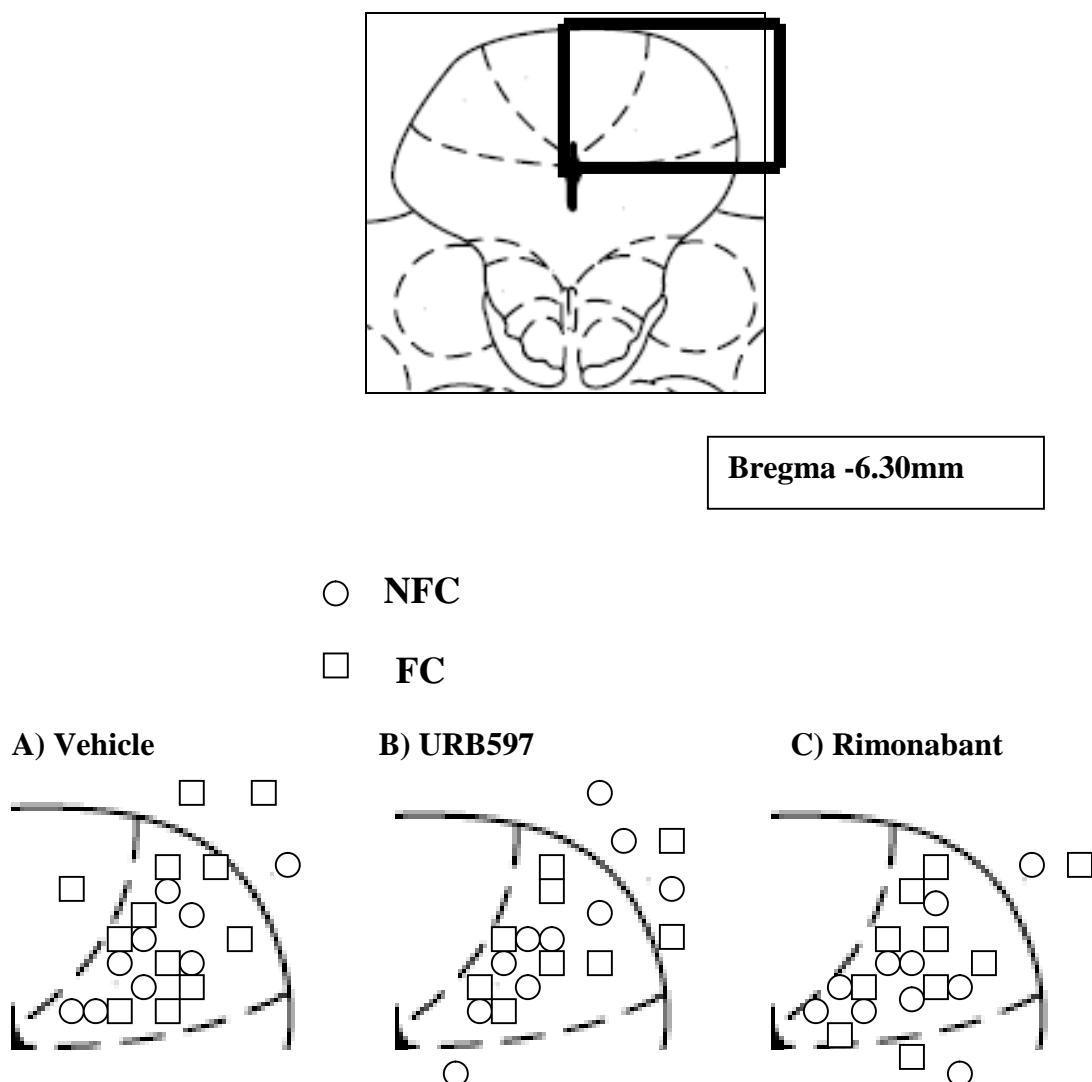
### **3.2.9 Statistical analysis**

The SPSS 17.0 statistical package was used to analyse all data and GraphPad Prism 5.0 was used for the construction of the graphs. Normality and homogeneity of data were assessed using Shapiro-Wilk and Levene test, respectively. Behavioural and neurochemical data were analysed using two-factor analysis of variance (ANOVA), with the factors being fear-conditioning and drug. Post-hoc pair-wise comparisons were made with Fisher's LSD when appropriate. Data were considered significant when P<0.05. Results are expressed as group means  $\pm$  standard error of the mean ( $\pm$  SEM).

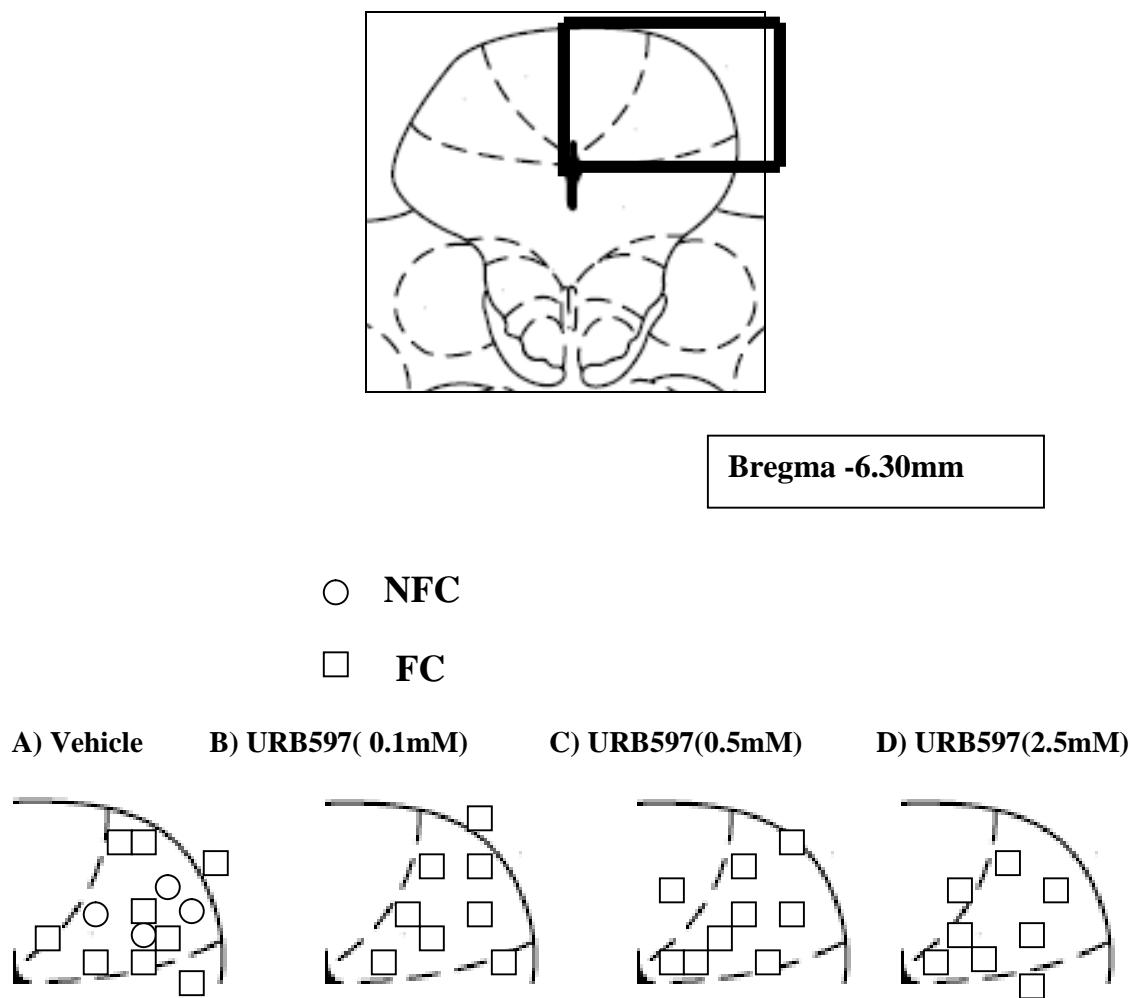
### 3.3 Results

#### 3.3.1 Histological verification of injector placement

In Experiments 1 and 2, eighty and seventy five percent of the injections, respectively, were placed within the borders of the right dlPAG (**Fig. 3.1 and 3.2**), with the remaining 20% and 25% positioned in the superior colliculus, ventral PAG or dorsomedial PAG. Only the results of experiments in which injections were correctly positioned in the right dlPAG were included in the analysis.



**Figure 3.1** Schematic depicting the sites of injection of (A) vehicle (100% DMSO) or (B) inhibitor of the catabolic enzyme FAAH, URB597 and (C) the CB<sub>1</sub> receptor antagonist/inverse agonist rimonabant in the right dorsolateral periaqueductal grey (dlPAG) in experiment 1. FC, fear-conditioned; NFC, non-fear conditioned. Adapted from Paxinos & Watson (1997)

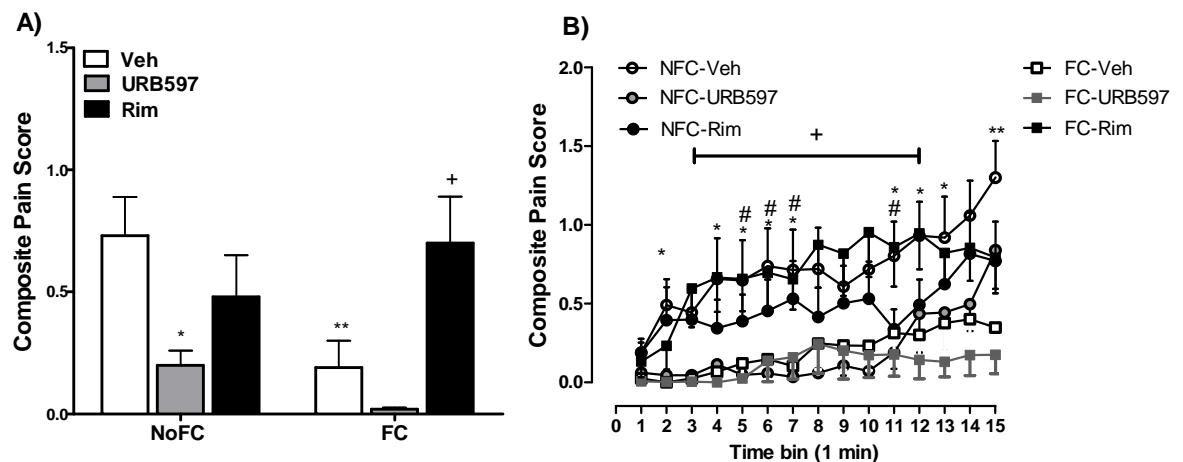


**Figure 3.2** Schematic depicting the sites of injection of (A) vehicle (100% DMSO) or (B) inhibitor of the catabolic enzyme FAAH, URB597 (0.1mM) (C) URB597(0.5mM) D) URB597(2.5mM) in the right dorsolateral periaqueductal grey (dlPAG) in experiment 2. FC, fear-conditioned; NFC, non-fear-conditioned. Adapted from Paxinos & Watson (1997)

### **3.3.2 Experiment 1**

#### **3.3.2.1 Effects of intra-dlPAG administration of rimonabant or URB597 on formalin-induced nociceptive behaviour and FCA**

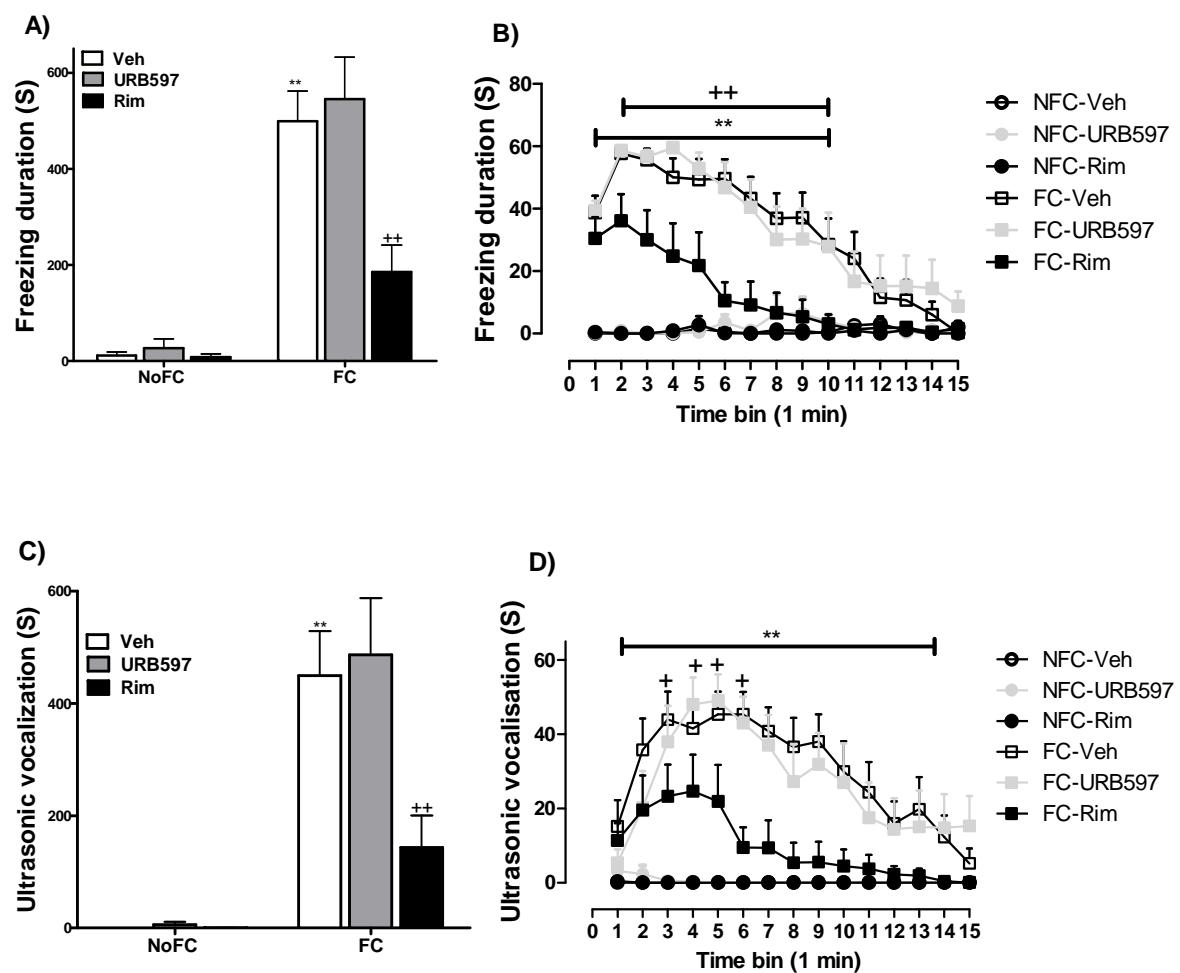
Intra-plantar injection of formalin produced robust licking, biting, shaking, flinching and elevation of the injected paw, behaviours which comprise the composite pain score (CPS) (**Fig. 2**). In addition, intra-plantar injection of formalin evoked oedema of the hind paw in both FC and NFC rats in a comparable manner (change in paw diameter: NFC-Veh  $1.35\text{mm} \pm 0.8$  vs. FC-Veh  $1.44\text{mm} \pm 0.16$ ). Neither rimonabant nor URB597 had a significant effect on formalin-evoked hind paw oedema when administered into the right dlPAG. Re-exposure of rats to the arena previously paired with footshock resulted in a significant reduction in formalin-evoked nociceptive behaviour, confirming expression of FCA (CPS: NFC-Veh *vs.* FC-Veh,  $p<0.01$ , **Fig. 3.3**). Analysis of the temporal profile of the nociceptive behaviour revealed that FCA was seen as early as 2min following re-exposure to conditioned stressor (**Fig. 3.3b**). Intra-dlPAG administration of rimonabant (Rim) did not alter formalin-evoked nociceptive behaviour in non-fear-conditioned rats but significantly attenuated FCA (FC-Veh *vs.* FC-Rim,  $p<0.05$ ), thus indicating a specific effect on FCA rather than on nociceptive behaviour *per se*. Intra-dlPAG administration of URB597 significantly reduced formalin-evoked nociceptive behaviour in non-fear-conditioned rats (CPS: NFC-Veh *vs.* NFC-URB,  $p<0.05$ ) when compared with vehicle-treated counterparts and showed a tendency to reduce formalin-evoked nociceptive behaviour in fear-conditioned rats (i.e. tendency to enhance FCA) although the latter effect failed to reach statistical significance.



**Figure 3.3** Effect of fear-conditioning and intra-dlPAG administration of rimonabant (Rim, 2mM) or URB597(0.1mM) on formalin-evoked nociceptive behaviour in rats during a 15min re-exposure to an observation chamber paired (FC)/not paired (NFC) 24h previously with footshock. A) Entire 15 min trial period :\*p<0.05, \*\*p<0.01 vs. NFC-Veh; +p<0.05 vs. FC-Veh (Fisher's LSD post hoc test following ANOVA: drug F(2,40) = 5.68, p<0.01; fear conditioning:  $F_{(1,40)}=1.58$ , p=0.216; drug x fear conditioning  $F_{(2,40)}=3.48$ , p<0.05); B) 1min intervals (Two way repeated measures ANOVA: time  $F_{(14,560)}=11.62$ , p<0.01, time x fear-conditioning  $F_{(14,560)}=2.87$ , p<0.01; time x drug  $F_{(4,80)}=1.449$ , p=0.23); Data expressed as Mean  $\pm$  SEM (n=6-9) \*p<0.05, \*\*p<0.01 NFC-Veh vs. FC-Veh; +p<0.05 FC-Veh vs. FC-Rim, #p<0.05 NFC-Veh vs. NFC-URB. CPS, composite pain score; FC, fear-conditioned; NFC, non-fear-conditioned; Veh, vehicle; Rim, rimonabant

**3.3.2.2 Effects of intra-dlPAG administration of rimonabant or URB597 on expression of conditioned fear behaviour in the presence of formalin-evoked nociceptive tone**

Fear conditioned rats receiving intra-dlPAG vehicle displayed significantly increased conditioned aversive behaviour as measured by the duration of freezing and 22 kHz ultrasonic vocalisation in formalin-treated rats when compared with non fear-conditioned, vehicle-treated counterparts (NFC-Veh *vs.* FC-Veh,  $p<0.01$ , **Fig. 3.4**). Intra-dlPAG administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, significantly reduced the duration of contextually-induced freezing and 22kHz ultrasonic vocalisation in the presence of formalin-induced nociceptive tone, compared with fear-conditioned rats receiving vehicle (FC-Veh *vs.* FC-Rim,  $p<0.01$ ). Intra-dlPAG administration of URB597 did not alter fear-related behaviours observed in the presence of nociceptive tone.



**Figure 3.4** Effects of fear-conditioning and intra-dlPAG administration of rimonabant (2mM) or URB597(0.1mM) on (A) total duration of freezing (ANOVA: drug  $F_{(2,40)}=8.07$ ,  $p<0.01$ ; fear conditioning  $F_{(1,40)}=80.8$ ,  $p<0.01$ ; drug x fear conditioning interaction  $F_{(2,40)}=6.16$ ,  $p<0.01$ ) \*\*  $p<0.01$  vs. NFC-Veh; ++  $p<0.01$  vs. FC-veh, (Fisher's LSD) B) 1min time bin- Freezing (Two way repeated measures ANOVA: time  $F_{(14,560)}= 20.46$ ,  $p<0.01$ , time x drug  $F_{(28,560)}=1.57$ ,  $p<0.05$ , time x fear conditioning  $F_{(14,560)}= 21.22$ ,  $p<0.01$ ) \*\* $p<0.01$  NFC-Veh vs. FC-Veh, + $p<0.05$ , ++ $p<0.05$  FC-Veh vs. FC-Rim (C) total duration of 22kHz ultrasonic vocalisation (ANOVA: drug  $F_{(2,40)}=4.25$ ,  $p<0.05$ ; fear conditioning  $F_{(1,40)}=47.02$   $p<0.01$ , drug x fear conditioning  $F_{(2,40)}=4.13$ ,  $p<0.05$ ) in formalin-injected rats during the 15min re-exposure to an observation chamber paired (FC)/not paired (NFC) 24h previously with footshock. \*\*  $p<0.01$  vs. NFC-Veh; ++  $p<0.01$  vs. FC-veh, (Fisher's LSD); D) 1min time bin- 22KHz US vocalization (Two way repeated measures ANOVA: time  $F_{(14,560)}=10.88$ ,  $P<0.01$ , time x fear conditioning  $F_{(14,560)}=11.12$ ,  $p<0.01$ ; time x drug  $F_{(4,80)}=1.57$ ,  $p=0.189$  \*\* $p<0.01$  NFC-Veh vs. FC-Veh, + $p<0.05$  FC-Veh vs. FC-Rim Data expressed as Mean  $\pm$  SEM (n=6-9). FC, fear-conditioned; NFC, non-fear-conditioned; Veh, vehicle; Rim, rimonabant;

### **3.3.2.3Effects of fear conditioning, rimonabant or URB597 on locomotor activity and defecation in formalin-treated rats**

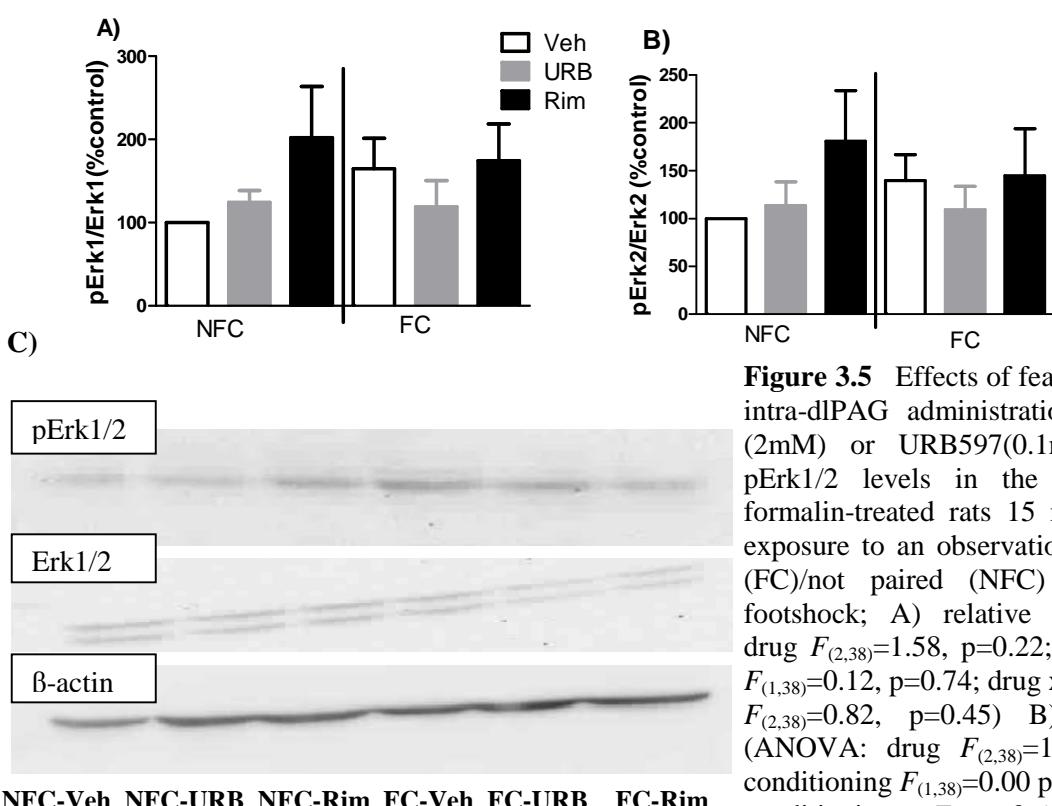
In rats which received intra-dlPAG vehicle, fear-conditioning significantly reduced the duration of locomotor activity measured as the sum of time spent rearing, grooming and walking and concurrently increased defecation when compared with the non-fear conditioned control group (NFC-Veh vs. FC-Veh,  $p<0.01$ ; **Table 3.1**). Neither rimonabant nor URB597 altered locomotor activity or defecation when administered into the dlPAG in fear-conditioned or non fear-conditioned rats when compared with respective vehicle-treated rats (Table 3.1).

Groups	Total activity (s)	Defecation(number of pellets)
NFC- Veh	205.25 $\pm$ 27.91	0.00 $\pm$ 0.00
NFC-URB	258.17 $\pm$ 22.00	0.00 $\pm$ 0.00
NFC- Rim	273.25 $\pm$ 53.00	0.00 $\pm$ 0.00
FC-Veh	80.44 $\pm$ 16.93**	3.25 $\pm$ 0.81**
FC-URB	91.71 $\pm$ 24.70	2.13 $\pm$ 0.48
FC-Rim	89.25 $\pm$ 19.76	2.00 $\pm$ 0.50

**Table 3.1** Effects of fear conditioning and intra-dlPAG administration of rimonabant or URB597 on locomotor activity and defecation;Effects of fear conditioning and intra-dlPAG administration of rimonabant (2mM) or URB597(0.1mM) on locomotor activity (measured as the sum of time spent rearing, grooming and walking) and defecation in formalin-treated rats during a 15 min re-exposure to an observation chamber paired (FC)/not paired (NFC) 24h previously with footshock. Two-way ANOVA locomotor activity: (fear conditioning  $F_{(1,29)}=23.3$ ,  $p< 0.01$ ; drug  $F_{(2,40)}=0.97$ ,  $p=0.38$  ; drug x conditioning  $F_{(2,40)}=0.53$ ,  $p=0.59$ ); defecation: (fear conditioning  $F_{(1,29)}=26.8$ ,  $p<0.01$ ; drug  $F_{(2,38)}=0.85$ ,  $p=0.38$ ; drug x conditioning  $F_{(2,38)}=0.25$  ,0.56); \*\* $p<0.01$  vs. corresponding NFC control); All data are expressed as Mean  $\pm$  SEM ( $n=6-9$ ). FC, fear-conditioned; NFC, non-fear conditioned; Veh, vehicle; Rim, rimonabant; URB, URB597

### 3.3.2.4 Effect of fear conditioning and rimonabant or URB597 on pErk1/2 levels in the right dlPAG of formalin-treated rats

In the presence of nociceptive tone, contextual fear conditioning did not have a statistically significant effect on levels of relative pErk1 and relative pErk2. However, a trend to increased levels of pErk1/2 was seen in FC rats receiving vehicle microinjection into the dlPAG when compared to non-fear conditioned counterparts. In fear-conditioned or non fear-conditioned rats, URB597 or rimonabant, had no significant effect on the expression of pErk1 and pErk2 in the right dlPAG when compared with vehicle-treated controls. Administration of rimonabant did produce an approximately 2-fold increase in expression of pErk1 and 1.75-fold ( $\pm 40\%$ ) increase in pErk2 in non fear-conditioned rats (NFC-Veh vs. NFC-Rim, **Fig. 3.5**), changes which did not reach statistical significance. This trend towards increased expression was not seen in fear-conditioned counterparts (FC-Veh vs. FC-Rim, **Fig. 3.5**).



**Figure 3.5** Effects of fear-conditioning and intra-dlPAG administration of rimonabant (2mM) or URB597(0.1mM) on relative pErk1/2 levels in the right dlPAG of formalin-treated rats 15 min following re-exposure to an observation chamber paired (FC)/not paired (NFC) previously with footshock; A) relative pErk1 (ANOVA: drug  $F_{(2,38)}=1.58$ ,  $p=0.22$ ; fear conditioning  $F_{(1,38)}=0.12$ ,  $p=0.74$ ; drug x fear conditioning  $F_{(2,38)}=0.82$ ,  $p=0.45$ ) B) relative pErk2 (ANOVA: drug  $F_{(2,38)}=1.16$ ,  $p=0.32$ ; fear conditioning  $F_{(1,38)}=0.00$   $p=0.99$ ; drug x fear conditioning  $F_{(2,38)}=0.63$ ,  $p=0.54$ ) C) Representative photomicrograph of pErk1/2, total Erk1/2 and the endogenous control  $\beta$ -actin; All data are expressed as Mean  $\pm$  SEM (n=6-9); NFC, non fear- conditioned; FC, fear conditioned; veh, Vehicle; URB, URB597; Rim, Rimonabant

**3.3.2.5 Effects of fear conditioning and intra-dlPAG administration of URB597 or rimonabant on monoamine levels in the RVM**

Neither fear-conditioning nor intra-dlPAG administration of URB597 or rimonabant, were associated with alterations in monoamine levels in the RVM (**Table 3.2**). In addition, analysis of DOPAC/DA and 5-HIAA/5-HT, measures of dopamine and serotonin turnover, respectively, revealed no differences between the treatment groups.

**Table 3.2: Effect of dlPAG microinjection of rimonabant or URB597 on RVM levels of monoamines and their metabolites**

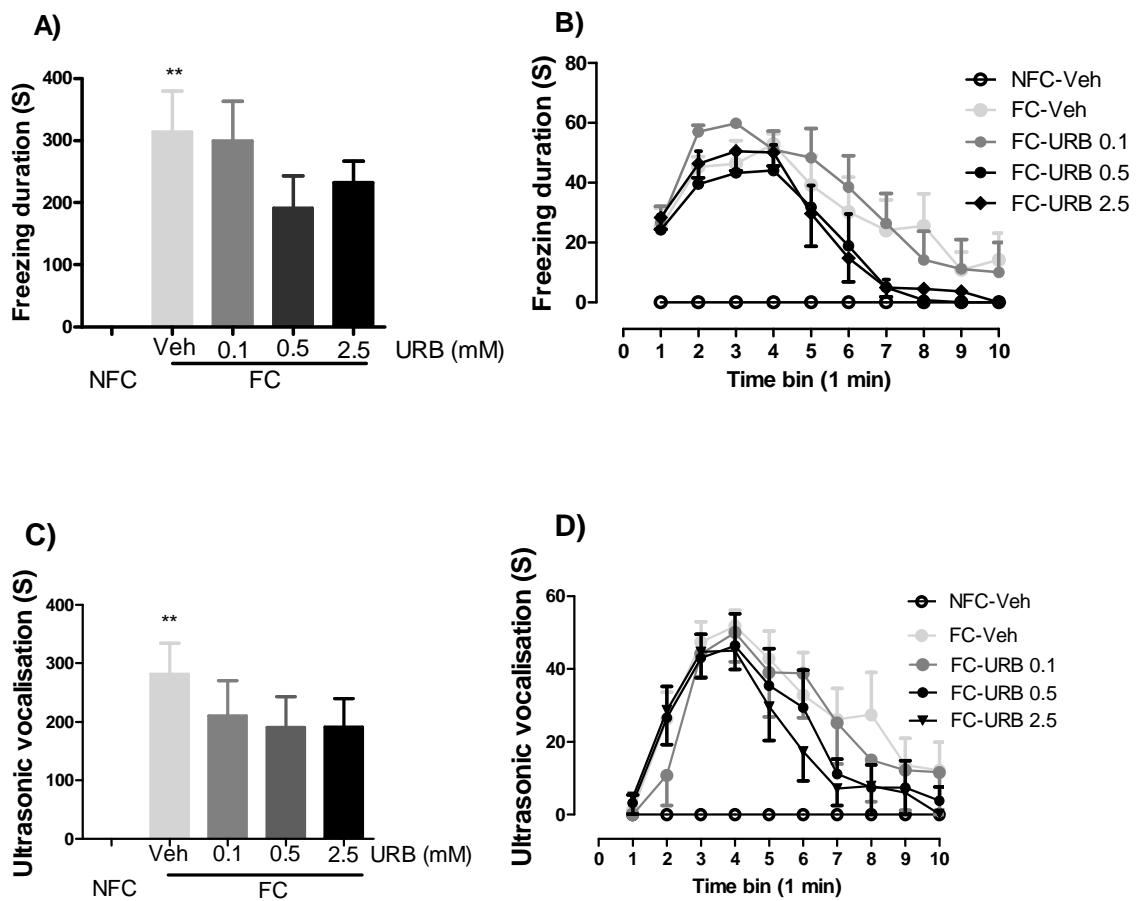
Groups	NA		DOPAC		DA		5-HIAA		5-HT		DOPAC/DA	5-HIAA/5-HT
	Mean± SEM		Mean± SEM		Mean± SEM		Mean± SEM		Mean± SEM		Mean± SEM	Mean± SEM
NFC-Vehicle	387.61	±129.48	110.39	±46.26	125.56	±27.37	1226.77	±257.69	756.34	±201.35	1.02	±0.30
NFC-URB597	445.38	±32.86	216.28	±26.78	133.88	±39.13	1275.43	±195.12	821.10	±96.08	1.05	±0.14
NFC-Rimonabant	518.77	±78.46	132.66	±42.52	140.30	±28.75	1335.86	±158.85	846.01	±111.67	0.86	±0.18
FC-Vehicle	464.43	±54.48	136.66	±16.33	82.68	±50.35	1186.43	±111.70	772.23	±95.87	0.88	±0.29
FC-URB597	430.64	±44.82	210.49	±27.06	104.93	±18.59	1149.84	±132.84	987.65	±125.12	0.89	±0.25
FC-Rimonabant	377.32	±50.93	304.81	±38.06	134.31	±42.32	1226.42	±211.03	725.75	±88.80	0.97	±0.23

Effect of dlPAG microinjection of rimonabant (2mM) or URB597 (0.1mM) on RVM levels of monoamines and their metabolites (expressed as ng/g tissue) or turnover in formalin-treated rats re-exposed to arena paired (FC)/not paired (NFC) 24h previously with footshock. NA (drug  $F_{(2,38)}=0.05$ ,  $p=0.95$ ; fear conditioning  $F_{(1,38)}=0.19$ ,  $p=0.66$ ); drug x fear conditioning  $F_{(2,38)}=1.19$ ,  $p=0.31$ ); DOPAC (drug  $F_{(2,38)}=0.48$ ,  $p=0.62$ ; fear conditioning  $F_{(1,38)}=0.82$ ,  $p=0.37$ ); drug x fear conditioning  $F_{(2,38)}=0.15$ ,  $p=0.86$ ); DA: (drug  $F_{(2,38)}=0.19$ ,  $p=0.83$ ; fear conditioning  $F_{(1,38)}=0.15$ ,  $p=0.69$ ); drug x fear conditioning  $F_{(2,38)}=0.41$ ,  $p=0.67$ ); 5-HIAA (drug  $F_{(2,38)}=0.11$ ,  $p=0.89$ ; fear conditioning  $F_{(1,38)}=0.38$ ,  $p=0.54$ ); drug x fear conditioning  $F_{(2,38)}=0.03$ ,  $p=0.97$ ); 5-HT (drug  $F_{(2,38)}=0.68$ ,  $p=0.51$ ; fear conditioning  $F_{(1,38)}=0.04$ ,  $p=0.84$ ); drug x fear conditioning  $F_{(2,38)}=0.65$ ,  $p=0.53$ ); DOPAC/DA (drug  $F_{(2,38)}=0.03$ ,  $p=0.97$ ; fear conditioning  $F_{(1,38)}=0.09$ ,  $p=0.76$ ); drug x fear conditioning  $F_{(2,38)}=0.19$ ,  $p=0.82$ ); 5-HIAA/5-HT (drug  $F_{(2,37)}=1.06$ ,  $p=0.36$ ; fear conditioning  $F_{(1,37)}=0.50$ ,  $p=0.48$ ); drug x fear conditioning  $F_{(2,37)}=0.29$ ,  $p=0.75$ ); Data presented as Mean ± SEM (n=6).

### **3.3.3 Experiment 2**

#### **3.3.3.1 Effect of intra-dlPAG administration of URB597 on expression of conditioned fear in the absence of nociceptive tone**

Fear-conditioned rats receiving administration of vehicle exhibited contextually-induced freezing behaviour and 22KHz ultrasonic vocalisation compared with non-fear conditioned vehicle-treated rats which displayed no contextually-induced freezing or 22 KHz ultrasound emission (NFC-Veh vs. FC-Veh,  $p<0.01$ ; **Fig. 3.6**). Intra-dlPAG administration of URB597 tended to decrease ultrasonic vocalization and freezing behaviour in a dose-dependant manner, however this effect failed to reach statistical significance. Further temporal analysis revealed an effect of time on ultrasound emission and freezing (Two way repeated measures ANOVA: Time  $F_{(9,144)} = 21.54$ ,  $p<0.01$ ). Post-hoc analysis demonstrated that the duration of both freezing and ultrasonic vocalisation reduced over the course of the trial, an effect not altered by intra-dlPAG administration of increasing concentrations of URB597.



**Figure 3.6** A) Effect of intra-dlPAG administration of increasing concentrations of URB597 (0.1, 0.5, 2.5mM) on duration of freezing (A) over 15 min trial, ( drug  $F_{(3,22)}=1.70$ ,  $p=0.19$ ; fear conditioning  $F_{(1,22)}=13.31$ ,  $p<0.01$ ) (B), over 1 min time bins (Two way repeated measures ANOVA: time  $F_{(9,216)}=9.75$ ,  $p<0.01$ , time x drug  $F_{(9,216)}=1.49$ ,  $p= 0.41$ , time x fear conditioning  $F_{(9,216)}= 2.51$ ,  $p<0.01$ ; and ultrasonic vocalisation (C) over 15 min trial (drug  $F_{(3,22)}=0.18$ ,  $p=0.91$ ; fear conditioning  $F_{(1,22)}=5.79$ ,  $p<0.05$  (D), over 1 min time bins (Two way repeated measures ANOVA: time  $F_{(9,225)}=6.45$ ,  $p<0.01$ , time x drug  $F_{(9,225)}=0.78$ ,  $p= 0.63$ , time x fear-conditioning  $F_{(9,225)}= 2.03$ ,  $p<0.05$  in rats re-exposed to context previously associated with foot shock; Data are expressed in Mean + SEM ( $n=6$ ); \*\*  $p<0.01$  vs. NFC (ANOVA followed by SNK); NFC, non fear conditioned; FC, fear conditioned; Veh, vehicle , URB, URB597

### **3.3.3.2 Effect of intra-dlPAG administration of URB597 on locomotor activity and defecation**

Fear-conditioned rats showed significantly decreased locomotor activity, measured as the sum of rearing, walking and grooming (NFC-Veh vs. FC-Veh, **Table 3.3**) when compared to non fear-conditioned rats. However, intra-dlPAG administration of different concentrations of URB597 did not have an effect on the fear-induced suppression of locomotor activity.

Groups	Total activity (s)		Defecation (number of pellets)	
NFC-Veh	300.33	$\pm 16.35$	1.67	$\pm 0.88$
FC-Veh	114.67	$\pm 32.11^*$	3.00	$\pm 0.44^*$
FC-URB597-0.1	106.00	$\pm 24.85$	2.29	$\pm 0.99$
FC-URB597-0.5	155.00	$\pm 21.89$	3.17	$\pm 0.79$
FC-URB597-2.5	172.00	$\pm 26.04$	4.17	$\pm 0.79$

**Table 3.3** Effect of dlPAG microinjection of various doses of URB597 on locomotor activity and defecation; Effect of dlPAG microinjection of various doses of URB597 (0.1, 0.5, 2.5mM) on locomotor activity (drug  $F_{(3,22)}=151$ ,  $p=0.23$ ; fear conditioning  $F_{(1,22)}=17.35$ ,  $p<0.01$ ) and defecation (drug  $F_{(3,22)}=0.98$ ,  $p=0.41$ ; fear conditioning  $F_{(1,22)}=4.9$ ,  $p<0.05$ ) in rats re-exposed to an arena previously paired(FC) or not paired(NFC) with foot shock. \* $p<0.05$  vs. FC-Veh; Data presented as Mean  $\pm$  SEM (n=6); (ANOVA followed by LSD post hoc test); NFC, non fear-conditioned; FC, fear-conditioned; Veh, vehicle

### **3.3.3.3 Effect of fear conditioning and intra-dlPAG administration of URB597 on monoamine levels in the RVM**

There was no effect of fear conditioning or intra-dlPAG administration of URB597 on monoamine levels in the RVM (**Table 3.4**). In addition, 5-HIAA/5-HT and DOPAC/DA a measure of serotonin and dopamine turnover, respectively was not affected by any of the treatments.

Groups	NA		DOPAC		DA		5HIAA		5HT		DOPAC/DA		5HIAA/5HT	
	Mean±SEM		Mean±SEM		Mean±SEM		Mean±SEM		Mean±SEM		Mean±SEM		Mean±SEM	
NFC-Veh	669.57	±91.22	137.17	±11.13	42.13	±1.54	1161.21	±123.51	925.57	±101.54	3.28	±0.37	1.26	±0.01
FC-Veh	551.45	±102.12	98.47	±8.60	38.66	±8.19	864.70	±113.42	930.13	±103.53	2.15	±0.21	1.00	±0.19
FC-URB597-0.1mM	643.73	±137.11	127.49	±23.40	37.24	±10.73	1183.50	±117.60	976.27	±143.01	3.23	±0.52	1.32	±0.17
FC-URB597-0.5mM	596.15	±91.03	111.98	±18.77	38.24	±10.37	1147.47	±127.86	849.35	±97.61	2.79	±0.29	1.37	±0.08
FC-URB597-2.5nM	697.11	±89.82	124.33	±24.52	42.37	±6.85	1314.72	±253.28	1096.66	±164.79	2.96	±0.23	1.21	±0.12

**Table 3.4:** Effect of dlPAG microinjection of URB597 on RVM levels of monoamines and their metabolites;Effect of dlPAG microinjection of URB597 (0.1, 0.5, 2.5mM) on RVM levels of monoamines and their metabolites (expressed as ng/g tissue) or turnover in rats re-exposed to arena previously associated with footshock. NA (drug  $F_{(3,22)}=0.36$ ,  $p=0.78$ ; fear conditioning  $F_{(1,22)}=0.43$ ,  $p=0.51$ ); DOPAC (drug  $F_{(3,22)}=0.48$ ,  $p=0.69$ ; fear conditioning  $F_{(1,22)}=1.37$ ,  $p=0.25$ ); DA: (drug  $F_{(3,22)}=0.06$ ,  $p=0.98$ ; fear conditioning  $F_{(1,22)}=0.05$ ,  $p=0.82$ ); 5HIAA (drug  $F_{(3,22)}=1.43$ ,  $p=0.26$ ; fear conditioning  $F_{(1,22)}=1.17$ ,  $p=0.29$ ); 5HT (drug  $F_{(3,22)}=0.67$ ,  $p=0.58$ ; fear conditioning  $F_{(1,22)}=0.00$ ,  $p=0.98$ ; DOPAC/DA drug  $F_{(3,19)}=1.95$ ,  $p=0.15$ ; fear conditioning  $F_{(1,22)}=4.35$ ,  $p=0.051$ ); 5HIAA/5HT (drug  $F_{(3,22)}=1.39$ ,  $p=0.27$ ; fear conditioning  $F_{(1,22)}=1.13$ ,  $p=0.29$ ); Data presented as Mean±SEM (n=6).

### **3.4 Discussion**

The present study demonstrated that the CB<sub>1</sub> receptor antagonist/inverse agonist rimonabant, injected directly into the right dlPAG, prevents conditioned fear-induced suppression of formalin-evoked nociceptive behaviour which results following re-exposure of rats to a context previously paired with aversive footshock (i.e. prevents fear-conditioned analgesia; FCA). This blockade of FCA by intra-PAG rimonabant was accompanied by an attenuation of conditioned fear responding in the presence of formalin-evoked nociceptive tone. In addition, acute intra-dlPAG administration of the endocannabinoid catabolism inhibitor, URB597, reduced formalin-induced nociceptive behaviour and showed a strong tendency to enhance FCA but had no effect on conditioned fear responding. Moreover, intra-dlPAG administration of URB597 at various doses did not alter the expression of conditioned fear-related behaviour in the absence of nociceptive tone. The expression of pErk1/2 in the dlPAG and monoamine levels in the RVM, were unaffected by FCA and its modulation by these drugs. Together, these results represent the first demonstration of an important role for the endocannabinoid system in the dlPAG in mediating analgesia induced by conditioned psychological stress/fear and in regulating fear expression during pain responding.

The suppression of formalin-evoked nociceptive behaviour observed here upon re-exposure to a context previously paired with footshock is similar in its nature and magnitude to previous reports demonstrating FCA using related or identical paradigms (Finn *et al.*, 2004; Helmstetter *et al.*, 1987; Roche *et al.*, 2007). Systemic (i.p.) administration of rimonabant has previously been shown to prevent FCA in rats (Finn *et al.*, 2004). Furthermore, recent work has demonstrated enhancement of FCA following systemic administration of the FAAH inhibitor URB597 (Butler *et al.*, 2008, Butler *et al.*, 2011), and blockade of this URB597-induced enhancement by rimonabant (Butler *et al.*, 2008). Bilateral administration of URB597 into the ventral hippocampus enhanced FCA in a CB<sub>1</sub>-dependent manner suggesting an important role for the endocannabinoid system in the ventral hippocampus in FCA (Ford *et al.*, 2011). However, intra-BLA administration of CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, did not affect FCA (Roche *et al.*, 2010). The present study demonstrates for the first time that direct administration of rimonabant into the right dlPAG prevents the fear-induced suppression of formalin-evoked nociceptive responding in rats without affecting the formalin-evoked response in non-fear-conditioned rats, confirming a

specific effect on FCA. In comparison, intra-dlPAG URB597 reduced nociceptive responding to formalin administration, an effect enhanced in the presence of conditioned fear. Hohmann and colleagues have previously demonstrated a key role for the endocannabinoid system in the dlPAG in mediating unconditioned stress-induced analgesia (Hohmann *et al.*, 2005). Their work demonstrated that intra-dlPAG administration of rimonabant attenuated unconditioned stress-induced analgesia while intra-dlPAG administration of FAAH or MAGL inhibitors enhanced unconditioned stress-induced analgesia (Hohmann *et al.*, 2005). In addition, systemic and site-specific injections of FAAH inhibitor, arachidonoyl serotonin (AA-5-HT) into either the dorsolateral PAG or RVM induced CB<sub>1</sub>-mediated enhancements of SIA (Suplita *et al.*, 2005). Our results here support these findings and extend our understanding by demonstrating a role for the endocannabinoid system in the dlPAG in analgesia resulting from exposure to Pavlovian conditioned psychological stress. Previous studies suggest differences with respect to the effects of rimonabant in the basolateral amygdala in unconditioned versus conditioned stress-induced analgesia (Connell *et al.*, 2006; Roche *et al.*, 2009; Roche *et al.*, 2007). It appears, however, that the dlPAG is a common neural substrate for endocannabinoid-mediated analgesia induced by exposure to either unconditioned or conditioned stress.

It seems reasonable to speculate that the fear-related increases in one or more of these lipids in the dlPAG may play a key role in mediating FCA, and that the rimonabant-induced blockade of FCA may be mediated by a blockade of the actions of AEA and/or 2-AG on CB<sub>1</sub> receptors in this region. Interestingly, our results (in chapter 1) revealed that AEA displayed a fear-related elevation in rats that received intra-plantar injection of formalin. These results suggest that elevations in AEA accompany the expression of FCA and that it may be the key endocannabinoid in the dlPAG mediating expression of FCA, possibly through activation of CB<sub>1</sub> receptors. Activation of CB<sub>1</sub> receptors in the PAG leads to subsequent disinhibition of output neurons and activation of the descending inhibitory pain pathway (de Novellis *et al.*, 2005; Vaughan *et al.*, 2000). However, it is also possible that alternative targets such as TRPV1 could mediate the effects of AEA in the dlPAG on FCA. There is good evidence for an important role of AEA activity at TRPV1 in the PAG in the regulation of both pain (Palazzo *et al.*, 2008; Maione *et al.* 2006) and aversion (Moreira *et al.*, 2009; Terzian *et al.*, 2009). However, Suplita *et al.* (2005) showed that TRPV1 was not involved in mediating unconditioned

stress-induced analgesia in rats. In addition, our results here demonstrate that intra-dlPAG administration of rimonabant prevented FCA completely rather than partially, suggesting that CB<sub>1</sub> receptor signalling in the dlPAG is necessary and sufficient for the expression of FCA in rats. Thus, the increased levels of AEA in the dlPAG in association with FCA (Chapter 1), the CB<sub>1</sub> receptor dependency of the FCA, and the URB597-mediated enhancement of FCA, together highlights the importance of the endocannabinoid system in analgesia associated with conditioned aversion. Furthermore, these data provide the first evidence of a role for the endocannabinoid system in the dlPAG in the modulation of tonic, persistent inflammatory pain by stress.

In the current experiment, we showed that intra-dlPAG administration of URB597 was antinociceptive in the formalin model of tonic persistent pain. This is in line with several previous reports showing the antinociceptive nature of this drug systemically (Hasanein, 2009; Jayamanne *et al.*, 2006; Kinsey *et al.*, 2009; Naidu *et al.*, 2010; Russo *et al.*, 2007) and intra-vIPAG (Maione *et al.*, 2006) in different animal models. Our finding here suggests that a FAAH substrate (e.g. AEA, OEA or PEA) in the dlPAG modulates nociceptive responding, probably by modulating the descending inhibitory pain pathway at this level. As the PAG tissue at the site of injection was used for histological verification of the injection site we were not able to confirm an increase in levels of endocannabinoids/NAEs following microinjection of URB597. In addition, indirect activation of TRPV1 by URB597 via the increase in anandamide cannot be ruled out (Di Marzo *et al.*, 2002; Maione *et al.*, 2006).

The PAG is critically involved in coordinating the defence response to aversive stimuli (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux *et al.*, 1988b; Schenberg *et al.*, 1990; Vianna *et al.*, 2003) and there is good evidence that the endocannabinoid system in the PAG plays an important role in the modulation of unconditioned (Bortolato *et al.*, 2006; Kathuria *et al.*, 2003; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Patel *et al.*, 2006a) and conditioned (Chhatwal *et al.*, 2007; Fendt *et al.*, 1996; Finn *et al.*, 2004; Lafenêtre *et al.*, 2007; Marsicano *et al.*, 2002; Resstel *et al.*, 2008) stress. The present experimental design enabled assessment of conditioned fear responding in the presence of formalin-evoked nociceptive tone. Our results revealed that attenuation of FCA by intra-dlPAG rimonabant was associated with a rimonabant-induced attenuation of

conditioned fear responding, measured as the duration of contextually induced freezing and 22kHz ultrasonic vocalization. This result corroborates previous reports of an inverse relationship between fear and pain responding (Butler *et al.*, 2008; Fanselow *et al.*, 1988; Finn *et al.*, 2004; Helmstetter, 1993; Roche *et al.*, 2009) and provides novel evidence that CB<sub>1</sub> receptors in the dlPAG may represent a key neural substrate regulating the reciprocal relationship shared by fear and pain. Previous work has shown that intra-PAG administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, AM251, blocked the anxiolytic effects of exogenous AEA (Moreira *et al.*, 2007) and prevented the attenuation of conditioned fear responses elicited by exogenous AEA (Ressell *et al.*, 2008) but failed to produce an effect on anxiety or fear responses by itself (Moreira *et al.*, 2007; Ressell *et al.*, 2008). It is possible that endocannabinoids in the dlPAG have a differential effect on fear responses depending on the presence or absence of nociception. Importantly, our data also demonstrate that while fear-induced suppression of nociceptive behaviour was prevented by intra-dlPAG rimonabant, fear-induced suppression of general locomotor/exploratory behaviour was not. These data suggest that the fear-induced suppression of formalin-evoked behaviour, and its blockade by intra-dlPAG rimonabant, represent specific effects on nociception rather than non-specific effects on general locomotor activity.

Intra-dlPAG administration of URB597 did not have any significant effect on contextually-induced conditioned fear behaviours, in the presence or absence of nociceptive tone. However, it should be noted that URB597 tended to decrease fear behaviour with increasing concentrations, although this effect failed to reach statistical significance. Previously, systemic administration of the FAAH inhibitor URB597 reduced anxiety-related behaviour in the rat elevated zero-maze and isolation-induced ultrasonic vocalisation tests (Kathuria *et al.*, 2003), elevated plus-maze (Hill *et al.*, 2007; Moise *et al.*, 2008; Naderi *et al.*, 2008; Naidu *et al.*, 2007; Patel *et al.*, 2006b), open field test (Moreira *et al.*, 2008) and the light dark test (Scherma *et al.*, 2008). Intra-dlPAG administration of URB597 (Lisboa *et al.*, 2008), AM404 or anandamide (Ressell *et al.*, 2008) also reduced expression of fear- or anxiety-related behaviour in contextual fear conditioning and Vogel conflict tests, respectively, in rats. However, differential modulation of anxiety at different doses of URB597 was noted following both systemic (Scherma *et al.*, 2008) and intra-PFC administration (Rubino *et al.*, 2008b) with low doses tending to be anxiolytic and higher doses anxiogenic or without effect. A recent

study found that systemic administration of URB597 did not produce robust anxiolytic effects when the aversiveness of testing procedures was increased (Haller *et al.*, 2009). In the same experiment, chlordiazepoxide had anxiolytic effects under all testing conditions suggesting that enhancing modulation of anxiety-like responses by endocannabinoids depends on the context of the test under examination.

In the present study, though not statistically significant, a strong trend towards increased expression of relative pErk1/2 in the dlPAG was seen in association with FCA. However, neither the reversal nor the enhancement of FCA by rimonabant and URB597 respectively was accompanied by alteration in expression of relative pErk1/2. Moreover, rimonabant showed a strong tendency to increase the expression of pErk1/2 in non fear-conditioned rats. Previously, expression of FCA was associated with increased relative pErk2 expression in the amygdala (Butler *et al* 2008). However, fear-conditioning had no significant effect on levels of relative pErk1/2 expression in the PAG of formalin-treated rats. In addition, URB597, significantly increased levels of relative pErk1/2 expression in the PAG of fear-conditioned, formalin-treated rats without any significant effect on relative pErk1/2 expression in non fear-conditioned formalin-treated rats. In chapter 2, both expression of conditioned fear and FCA were accompanied by increased expression of Erk1 in the right BLA. Direct comparison between these studies is not possible as the time of tissue extraction, the route of drug administration and the region investigated are different. Furthermore, Butler *et al* (2008) showed that both rimonabant-mediated attenuation and URB597-mediated enhancement of FCA was associated with reduced pErk2 in the amygdala, raising questions as to the role of pErk1/2 in the amygdala. Similarly, the present study demonstrates that intra-dlPAG rimonabant in non-fear conditioned formalin injected rats (with high CPS score) showed a trend towards increased expression of pErk1/2 in a similar manner to that seen in rats expressing FCA arguing against a causal role of pErk1/2 in the dlPAG in FCA. It should be noted that this is a single time point study and changes in expression of pErk1/2 could occur at a different time point and as such a possible role of pErk1/2 cannot be definitively ruled out. Indeed, as presented in chapter 2, measurement of relative pErk1/2 levels at 3min time point, when expression of FCA is maximal, suggested that pErk1/2 expression in the right dlPAG tended to increase and Erk1 increased in the right BLA. It is also possible that endocannabinoid-mediated FCA engages other intracellular molecules such as zif268 which is known to be increased

following CB<sub>1</sub> receptor activation (see chapter 4). Thus, future studies are needed to determine the role of such molecules in this CB<sub>1</sub>-dependent form of FCA.

Antinociception appears to be mediated at least in part by a pathway that projects from the PAG to the RVM. It is postulated that the PAG exerts this inhibitory effect on spinal nociceptive processing through the activation of descending serotonergic and noradrenergic pathways that arise from the RVM and pontine noradrenergic nuclei (Millan, 2002; Odeh *et al.*, 2001). In addition, it has been suggested that cannabinoids produce antinociception, in part, by modulating descending noradrenergic systems (Gutierrez *et al.*, 2003). The present study investigated the extent to which endocannabinoids in the PAG modulate monoamines in the RVM via the PAG-RVM projections. The data demonstrated no alterations in monoamine levels in the RVM associated with FCA or intra-dlPAG administration of cannabinoid ligands. This is in contrast to previous studies demonstrating FCA-associated changes in monoamine levels in several brain regions such as the PAG, thalamus and cerebellum (Finn *et al.*, 2006; Roche *et al.*, 2007) but differences in methodology, and in particular the presence of an intracerebral cannula and intra-dlPAG microinjection of vehicle, may account for this discrepancy. To our knowledge effects of PAG endocannabinoid modulation on RVM monoamines has not been investigated. Previously, intra-vIPAG administration of AA-5-HT, inhibitor of FAAH, increased endocannabinoid levels in the PAG, induced analgesia and prevented the changes in RVM ON and OFF cells firing activity induced by intra-plantar formalin in a CB<sub>1</sub>-dependent manner, indicating a role for endocannabinoids in this PAG-RVM pain pathway (de Novellis *et al.*, 2008). In the same experiment, levels of monoamines in the RVM were not assessed; however, AA-5-HT increased the firing activity of locus coeruleus neurons and reversed the formalin-induced increase in locus coeruleus noradrenergic cell activity in a CB<sub>1</sub>-dependent manner suggesting an endocannabinoid-dependent effect on descending monoaminergic pathway (PAG-locus coeruleus-spinal cord) activity (de Novellis *et al.*, 2008). It is possible that these locus coeruleus changes may be mediated by projections from the RVM. However, whether or not this interaction between the vIPAG and RVM occurs between the dlPAG and RVM is not known. It is also possible that endocannabinoids in the PAG engage non-monoaminergic neurons projecting to the RVM such as, GABAergic (Morgan *et al.*, 2008), opioidergic (Wang *et al.*, 2002) and glutametergic (Drew *et al.*, 2009).

In conclusion, the results reported here provide evidence to support the contention that the endocannabinoid system in the dlPAG is a key neural substrate mediating analgesia expressed during or following exposure to stress, including that evoked by Pavlovian conditioned fear to context. Pharmacological blockade of CB<sub>1</sub> receptors in the dlPAG prevented FCA in rats and reduced fear responding in the presence of nociceptive tone, whereas FAAH inhibition by URB597 tended to enhance FCA and reduced nociception without affecting expression of conditioned fear *per se*. Furthermore, the results suggest that pErk1/2 expression in the dlPAG is unlikely to play a causal role in the expression of endocannabinoid-mediated FCA. Neither endocannabinoid-mediated FCA nor modulation of endocannabinoids in the PAG was associated with alterations in RVM monoamine levels or turnover under the conditions investigated. Together these data suggest a key role for the endocannabinoid system in the dlPAG in mediating endogenous analgesia and modulating tonic persistent inflammatory pain states. These data advance our understanding of underlying neurochemical and molecular mechanisms mediating pain, fear and FCA.

**Chapter 4: Investigation of novel molecular correlates of endocannabinoid-mediated fear-conditioned analgesia**

#### **4.1 Introduction**

Pain and fear are defence responses which are initiated upon exposure to noxious or aversive stimuli, respectively. Induction of FCA in rodents provides a useful model with which to study the physiology of the endogenous analgesic system. To date, the study of FCA has focused largely on the neurochemical and receptor mechanisms underpinning this form of potent endogenous analgesia (Finn *et al.*, 2004; Harris and Westbrook, 1994, Fanselow and Helmstetter, 1988). Increased understanding of the molecular mechanisms/correlates of FCA is equally important, both on a fundamental physiological and potential therapeutic basis.

The endocannabinoid system is an important modulator of neural functions such as the control of fear- and pain-related behaviour (Finn, 2010; Guindon *et al.*, 2009b; Moreira *et al.*, 2009b). The neural substrates and pathways involved in cannabinoid-mediated modulation of both endogenous analgesia and aversion (fear, anxiety) overlap, suggesting the endocannabinoid system as an important common denominator. Neural projections from the amygdala, PAG and RVM constitute key components of the descending inhibitory pain pathway (Basbaum *et al.*, 1984a; Behbehani, 1995; Heinricher *et al.*, 2009; Hopkins *et al.*, 1978; Oka *et al.*, 2008) and fear system (Amorapanth *et al.*, 1999; Carrive *et al.*, 1999; Davis *et al.*, 1994; Holstege *et al.*, 1996; LeDoux, 2000). Endocannabinoids in these brain regions activate the descending pathway via GABA-mediated disinhibition (Szabo *et al.*, 2005) to inhibit impulse transmission at the level of DHSC. Thus far, several studies have demonstrated a role for the endocannabinoid system in SIA, with key roles for the PAG (Hohmann *et al.*, 2005; Suplita *et al.*, 2005), amygdala (Connell *et al.*, 2006), RVM (Suplita *et al.*, 2005) and DHSC (Suplita *et al.*, 2006). However little is known about the molecular mechanisms involved in endocannabinoid-mediated regulation of SIA/FCA. Activation of the CB<sub>1</sub> receptor increases expression of other intracellular molecules such as *zif268* (Bouaboula *et al.*, 1995), c-Fos (Patel *et al.*, 1998) and JNK1 and JNK2 (Rueda *et al.*, 2000).

Both fear and chronic pain states are accompanied by long-term changes in neuronal activity. Long term potentiation (LTP) in dorsal horn and brain neurons following peripheral injury may be one mechanism whereby acute pain can be transformed into a long-term pain (Ji *et al.*, 2003; Woolf *et al.*, 2000; Zhuo *et al.*, 2002). The acquisition and consolidation of fear memory is also known to involve LTP (Maren, 1999; Schafe *et al.*, 2001). *zif268* is a plasticity-related gene that encodes for *zif268* (also called NGFI-A, Krox24, Erg1 or tis-8). It is a zinc finger transcription factor that plays a critical role in coupling extracellular signals to changes in cellular gene expression (O'Donovan *et al.*, 1999). The expression of *zif268* has been closely correlated with the induction of hippocampal (Cole *et al.*, 1989; Wisden *et al.*, 1990; Worley *et al.*, 1993) and spinal LTP (Rygh *et al.*, 2006). Increased neuronal expression of *zif268* in the superficial dorsal horn was shown to be important in the initiation and long-term maintenance of inflammatory hyperalgesia (Rygh *et al.*, 2006). *zif268* expression is increased in response to various noxious stimuli in the DHSC (Delander *et al.*, 1997; Otahara *et al.*, 2003; Rahman *et al.*, 2002) and the lateral and medial thalamus (Pearse *et al.*, 2001) and increased in brain regions, such as the hippocampus and anterior cingulate cortex following amputation injury (Wei *et al.*, 1999; Wei *et al.*, 2000).

*zif268* expression is also associated with neural plasticity during the formation of fear memory in the hippocampus (Lonergan *et al.*, 2010) and within specific regions of the hippocampus and amygdala during fear memory retrieval (Hall *et al.*, 2001). Recent studies have demonstrated that this inducible-transcription factor is rapidly induced in the lateral nucleus of the amygdala following fear conditioning (Hall *et al.*, 2000; Malkani *et al.*, 2000; Ressler *et al.*, 2002; Rosen *et al.*, 1998a) and blocked following disruption of fear conditioning by the anxiolytic drug, diazepam (Malkani *et al.*, 2000). Furthermore, the acquisition of conditioned fear to context was associated with enhanced *zif268* expression in the basolateral amygdala (Perez-Villalba *et al.*, 2008). Moreover, expression of *zif268* in the amygdala is functionally important to contextual fear memory consolidation as infusions of *zif268* antisense into the amygdala prior to contextual fear conditioning disrupt fear memory consolidation (Malkani *et al.*, 2004).

The serum- and glucocorticoid-inducible kinase-1 (*sgk1*) is another ubiquitously expressed plasticity-related gene involved in a number of physiological functions such

as the regulation of transport, hormone release, neuroexcitability, cell proliferation, and apoptosis (Lang *et al.*, 2006). It is a serine and threonine protein kinase activated by the MAPK/Erk signalling pathway (Lee *et al.*, 2006) and known to activate many downstream molecules, including cAMP response element-binding protein (David *et al.*, 2005). Compelling evidence suggests that *sgk1* contributes to the regulation of diverse cerebral functions (e.g. memory consolidation, fear retention) and the pathophysiology of several cerebral diseases (e.g. depression) (Lang *et al.*, 2010). Recently, using a genome-wide micro-array profiling technique, *sgk1* has been shown to be upregulated in the DHSC after induction of peripheral inflammation (Geranton *et al.*, 2007). Furthermore, antisense knock-down of *sgk1* delayed the onset of inflammatory hyperalgesia by at least 24h, suggesting a novel role of this plasticity-related gene in the DHSC in the induction of inflammatory pain state (Geranton *et al.*, 2007). *sgk1* has also been implicated in learning and memory and synaptic plasticity (Ma *et al.*, 2006; Tsai *et al.*, 2002). It has been demonstrated that *sgk1* phosphorylation was increased after contextual fear conditioning in the hippocampus (Lee *et al.*, 2007). In addition, transient transfection of hippocampal neurons with the dominant negative mutant of *sgk*, *sgkS78A*, impaired, whereas, transfection of the constitutively active *sgk*, *sgkS78D*, enhanced fear retention (Lee *et al.*, 2007). Thus, both *sgk1* and *zif268* play important roles in neuronal plasticity in the dorsal horn and supraspinal substrates and may be candidate genes for modulating synaptic plasticity in pain states. The extent to which these molecular mediators of nociception and fear may be subject to supraspinal control following fear-induced activation of descending pain pathways is, however, unknown and was a key aim of the work presented in this chapter.

Cannabinoid receptor activation by agonists such as CP-55940 (Bouaboula *et al.*, 1995a), HU210 (Graham *et al.*, 2006) and THC (Valjent *et al.*, 2001) induces the expression of the immediate-early gene *zif268* in different cell lines expressing the CB<sub>1</sub> receptor. The signal transduction pathway between the CB<sub>1</sub> receptor and *zif268* is sensitive to the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant (Bouaboula *et al.*, 1995a). In addition, using quantitative *in situ* hybridization, Mailleux *et al.*, demonstrated that acute systemic administration of THC increases the expression of *zif268* in the cingulate cortex and the fronto-parietal cortex and caudate-putamen of the adult rat (Mailleux *et al.*, 1994). However, chronic treatment with THC impairs spatial

memory and reduces *zif268* expression in the mouse forebrain (hippocampus and prefrontal cortex) (Boucher *et al.*, 2009). Furthermore, CB<sub>1</sub> receptor stimulation with cannabinoid receptor agonists activated Erk and induced the transcription factor *zif268* in a Mek-Erk pathway-dependent manner (Graham *et al.*, 2006; Valjent *et al.*, 2001). To our knowledge, the effect of cannabinoid receptor activation on the expression of *sgk1* is unknown. Thus, it appears that CB<sub>1</sub> receptor activation engages *zif268* both *in vitro* and *in vivo*. However, the extent to which expression of *zif268* and/or *sgk1* is involved in endocannabinoid-mediated FCA is unknown.

The aims of these experiments were:

- to investigate whether behavioural expression of FCA in rats is associated with altered expression of *sgk1* or *zif268* in key supraspinal components of the descending inhibitory pain pathway (amygdala, PAG and RVM) or in the DHSC
- In addition, the role of the CB<sub>1</sub> receptor in the expression of *sgk1* and *zif268* in the dorsal horn during the expression of FCA was examined. This was achieved by examining the effect of systemic administration of the CB<sub>1</sub> receptor antagonist, AM251, on FCA and associated alterations in the expression level of *sgk1* and *zif268* in the DHSC

The work tests the hypothesis that formalin-ivoked nociception is associated with increased expression of *sgk1* and *zif268* mRNA in key regions such as amygdala, PAG, RVM and DHSC and that conditioned fear attenuates this increased expression in a CB<sub>1</sub>-dependent manner.

## **4.2 Methodology**

### **4.2.1 Animals**

Experiments were carried out on adult male Lister-Hooded rats (225–310g; Charles River, Kent, UK) maintained at a constant temperature ( $21 \pm 2^{\circ}\text{C}$ ) under standard lighting conditions (12:12h light:dark, lights on from 07.00 to 19.00h). All experiments were carried out during the light phase between 08.00h and 17.00h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

### **4.2.2 Drug preparation**

AM251 (1-[2,4-dichlorophenyl]-5-[4-iodophenyl]-4-methyl-N-[piperidin-1-yl]-1H-pyrazole-3-carboxamide; Ascent Scientific, UK) was prepared on day of use to a concentration of 1mg/ml in ethanol:cremophore:saline (1:1:18).

### **4.2.3 Experimental procedure**

The FCA paradigm was essentially as described previously (Butler *et al.*, 2008; Finn *et al.*, 2004; Finn *et al.*, 2006; Roche *et al.*, 2009; Roche *et al.*, 2007) and is described in Chapter 2 (Section 2.2).

#### **4.2.3.1 Experiment 1**

The test phase commenced 23.5h following fear conditioning when the subjects received an intra-plantar injection of 50 $\mu\text{L}$  formalin (2.5% in 0.9% saline) or saline (0.9%) into the right hind-paw under brief isoflurane anaesthesia (3% in O<sub>2</sub>; 0.5L/min). Rats were returned to their home cage for a further 30min, after which time they were re-exposed to the perspex observation chamber (to which they had been exposed during the conditioning phase) for 30 minutes. A group of formalin-treated rats that had received footshock in a different context (light: bright (lux, 200) vs. dimmed (lux, 30); smell: acetic acid (0.5%) vs. Milton:tap water (1:5); chamber cover: transparent vs. dark and background noise tone: 80dB vs. none) but were re-exposed to the same chamber as the other groups was also included. This group was included to determine whether any changes detected (behavioural or molecular) during re-exposure to the context are likely

to be associated with conditioned fear *per se* or with footshock exposure on the previous day.

This design resulted in five experimental groups: fear-conditioned + saline (FC-Sal); fear-conditioned + formalin (FC-Form); non fear-conditioned + saline (NFC-Sal); non fear-conditioned + formalin (NFC-Form) and fear-conditioned in a different context + formalin (FCD-Form). Behaviours were recorded for 30min with the aid of a video camera located beneath the observation chamber. The 30–60min post-formalin interval was chosen on the basis of previous studies demonstrating robust suppression of formalin-evoked nociceptive behaviour upon re-exposure to an aversively conditioned context during the second phase formalin response (Finn *et al.*, 2004; Finn *et al.*, 2006; Rea *et al.*, 2009a; Roche *et al.*, 2009; Roche *et al.*, 2007) and changes in gene expression reported during this time period post formalin injection or fear memory test (Lee *et al.*, 2007; Rygh *et al.*, 2006; von Hertzen *et al.*, 2005).

At the end of the test trial, rats were sacrificed, brains quickly removed and discrete brain regions (PAG, amygdala, RVM) and the L4–L6 segment of the ipsilateral and contralateral (relative to formalin injection) dorsal horn were gross dissected out on an ice-cold plate. Dissected tissues were immediately frozen on dry ice and subsequently stored at -80°C until further processing.

#### **4.2.3.2 Experiment 2**

The test phase commenced 23.5h following fear conditioning when rats received an intraplantar injection of 50µL formalin (2.5% in 0.9% saline) into the right hind-paw under brief isoflurane anaesthesia (3% in O<sub>2</sub>; 0.5L/min). At the same time, rats received intra-peritoneal AM251 (3mg/kg) or vehicle (ethanol: cremophore: saline; 1:1:18) at an injection volume of 3ml/kg. The dose of AM251 was chosen based on pilot studies from our group demonstrating that AM251 administered systemically at this dose attenuated FCA. Rats were returned to their home cage for a further 30min, after which time they were re-exposed to the perspex observation chamber (to which they had been exposed during the conditioning phase) for 30 min. This design resulted in four experimental groups: fear-conditioned + vehicle (FC-Veh); fear-conditioned + AM251 (FC-AM251); non fear-conditioned + vehicle (NFC-Veh) and non fear-conditioned +

AM251 (NFC–AM251). Behaviours were recorded for 30min with the aid of a video camera located beneath the observation chamber. At the end of the test trial, rats were sacrificed, brains quickly removed and frozen on dry ice and the L4–L6 segment of the ipsilateral and contralateral (relative to formalin injection) DHSC were gross dissected out on an ice-cold plate. Dissected tissues were immediately frozen on dry ice and subsequently stored at -80°C until further processing.

#### **4.2.4 Behavioural analysis**

Behaviour was analysed using the Observer XT 7.0 software package (Noldus Technology, Wageningen, the Netherlands), which allowed for continuous event recording over the duration of the trial as described in Chapter 2 (Section 2.2).

#### **4.2.5 Real time quantitative polymerase chain reaction (RT-qPCR)**

RVM and ipsilateral DHSC tissues from experiment 1 were processed and ran in the laboratory of Professor Stephen Hunt at University College London while the remaining tissues from experiments 1 and 2 were processed and ran at NUI Galway using similar methodology except that the PCR machine which was used in London was a DNA Engine System (Bio-Rad, Hercules, CA) and did not require reference dye (ROX) while the machine at NUI Galway was an Applied Biosystems 7500 system and was used with ROX reference dye.

##### **4.2.5.1 RNA extraction**

Total RNA was extracted from homogenized tissue using an acid phenol extraction method using trizol reagent (Bioscience, Dublin, Ireland), a Qiagen RNeasy mini kit and QIAshredder columns (Qiagen, UK). Tissue was homogenised manually using a dounce homogeniser in a 2ml glass tube containing 700 µl trizol and the homogenate transferred to QIAshredder columns. The eluent was treated with 140µl of chloroform, vortex mixed and left to stand for 3 min at room temperature and then centrifuged for 15 min (13,000 rpm at 4 °C). This resulted in 3 layers: top- translucent aqueous layer; middle- yellowish lipid layer and bottom- pinkish organic solvent layer. Only the aqueous layer was collected carefully to avoid contamination and was treated with (0.53 x sample volume)µl of 100% molecular grade ethanol before being transferred to RNeasy columns to be purified and concentrated. After a wash with 400µl washing

buffer (RW1, Qiagen, UK), the RNA samples were treated with 80 $\mu$ l DNase I (10 $\mu$ l [1500kunits/550 $\mu$ l] DNase I + 70 $\mu$ l RDD reaction buffer for DNase I [Qiagen, UK]) at room temperature for 15min to remove any DNA from the sample. The DNase enzyme cleaves DNA non-specifically. Samples were then serially washed using washing buffers (350 $\mu$ l RW1, 2x 500 $\mu$ l RPE (RPE, Qiagen, UK) and RNA was eluted in 20 $\mu$ l of RNase free water (Sigma, Dublin, Ireland). The quantity, purity and quality of RNA were assessed using nanodrop (ND-1000, Nanodrop, Labtech International, Ringmer, UK). RNA quantity was determined by measuring optical density (OD) at 260nm. RNA quality was determined by measuring the ratio OD<sub>260</sub>/OD<sub>280</sub> where a ratio of approximately 1.8-2.1 was deemed indicative of pure mRNA. All mRNA samples showed OD<sub>260</sub>/OD<sub>280</sub> ratios between 1.75 and 2.2 on the Nanodrop. mRNA samples were kept at -80°C prior to cDNA synthesis.

#### **4.2.5.2 cDNA synthesis**

RNA samples were equalised to 3,000ng/11 $\mu$ l using RNase free water. Equal amounts of total RNA (3,000ng in 11 $\mu$ l) from each sample were then reverse transcribed into complementary DNA (cDNA) as follows. Target RNA and primers were combined and denatured by addition of 11 $\mu$ l of RNA and 0.8 $\mu$ l random nanomers (Sigma, Dublin, Ireland), 0.2 $\mu$ l oligo(dT)15 primers (Medical supply Co, Dublin, Ireland) and 1 $\mu$ l PCR Nucleotide mix (dNTP mix, Medical supply Co, Dublin, Ireland) and incubation at 65°C for 5 min. The 13  $\mu$ l of target RNA mix was then mixed with 4 $\mu$ l of 1<sup>st</sup> strand buffer (Bioscience LTD, Dublin, Ireland), 1 $\mu$ l of DTT (Bioscience LTD, Dublin, Ireland), 1 $\mu$ l of recombinant ribonuclease inhibitor (RNaseOUT, Bioscience LTD, Dublin, Ireland) and 1 $\mu$ l of SuperScript III Reverse Transcriptase (Bioscience LTD, Dublin, Ireland) to a total reaction volume of 20 $\mu$ l. Two negative control reverse transcription reactions were included where reverse transcriptase or RNA templates were replaced with nuclease-free water. Samples were run on a PCR machine/thermocycler (MJ Research, INC, USA) using steps below:

Annealing: 25 °C for 5min

Extension: 50 °C for 50min

Inactivation: 70 °C for 15min

cDNAs were kept at -20°C until required for quantification by RT-PCR.

#### **4.2.5.3 cDNA amplification**

The cDNA was used as the template for RT-qPCR, which was performed on the AB7500 PCR system (Applied Biosystems 7500) using SYBR Green RT-PCR Master Mix (SYBR Green Jumpstart Taq Ready Mix, Sigma, Dublin, Ireland) with each gene-specific primer (see Table 4.1 for primer sequences). Previously prepared cDNA samples were diluted 1:4 (except RVM which was not diluted as the mRNA yield was lower) and each diluted sample was pipetted onto a MicroAmp<sup>TM</sup> optical 96 well plate (Applied Biosystems, Dublin, Ireland). cDNA was amplified in a reaction run in triplicate and contained 1µl of cDNA template, 1µl of reverse primer (concentration:  $\beta$ -actin and zif268 primers 2.5mM; GAPDH and sgk1 primers 5.5mM), 1µl forward primer (concentration:  $\beta$ -actin and zif268 primers 2.5mM; GAPDH and sgk1 primers 5.5mM), 12.5µl of SYBR green, 9.25µl RNase free water and 0.25µl reference dye (ROX) (in the kit with SYBR Green) to give a final reaction volume of 25µl in a three-step cycling program. Control cDNA samples (obtained without transcriptase or RNA) were always included, as well as a control without any cDNA template. Plates were covered with adhesive plate covers and spun at 1000g for 30 seconds to ensure complete mixing. Cycling parameters were stage 1: 94<sup>0</sup>C for 2min to activate DNA polymerase, then stage 2: 94<sup>0</sup>C for 15s to denature DNA, annealing temperature 60<sup>0</sup>C for 1min, and a final extension step of 72<sup>0</sup>C for 1min in which fluorescence was acquired. Stage 2 was repeated 40 times. Efficiencies of PCR were calculated from curves generated from the amplification and analysis of serially diluted cDNA. Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems 7500 SDS Software 1.3.1. Reactions were performed for 4-5 biological replicates and Ct values were normalized to the housekeeping/reference genes  $\beta$ -actin or GAPDH. Similar results were obtained with the two reference genes used for normalization. The data shown in the figures (section 4.3) correspond to the normalization using the GAPDH gene. The relative expression of target genes to GAPDH was calculated by using the 2<sup>ΔCt</sup> method. In this method,  $\Delta C_t = C_t$  for the reference gene in the test sample –  $C_t$  for the target gene in the test sample. The 2<sup>ΔCt</sup> values for each sample were then expressed as a percentage of the mean of the 2<sup>ΔCt</sup> values for the control group (NFC-Sal). In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined. The specificity of the amplified products was determined by melting curve analysis (melting

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parameters: 1 cycle of 95<sup>0</sup>C for 15s, 60<sup>0</sup>C for 1min and 95<sup>0</sup>C for 15s where fluorescence was measured).

**Table 4.1 Sequence of primers for RT qPCR**

<b>Gene</b>		<b>Primer sequence</b>	<b>Melting temperature(°C)</b>
<i>β-actin</i>	forward	5'-AGATTACTGCCCTGGCTCCTA-3'	63.8
	reverse	5'-AGGATAGAGCCACCAATCCAC-3'	64.1
<i>GAPDH</i>	forward	5'-ACTCTACCCACGCGGCAAGTTC-3'	63.1
	reverse	5'-GGTGGTGAAGACGCCAGTAG-3'	64.7
<i>sgk1</i>	forward	5'-GGGCTGTCTTGATGAGATGC-3	63.6
	reverse	5'-GTGCCTTGCTGAGTTGGTG-3'	64.7
<i>zif268</i>	forward	5'-ACCCACCTTCCTACTCCAA-3'	68.9
	reverse	5'-TGATAGTGGATAGTGGAGTGTA-3'	69

#### **4.2.6 Statistical analysis**

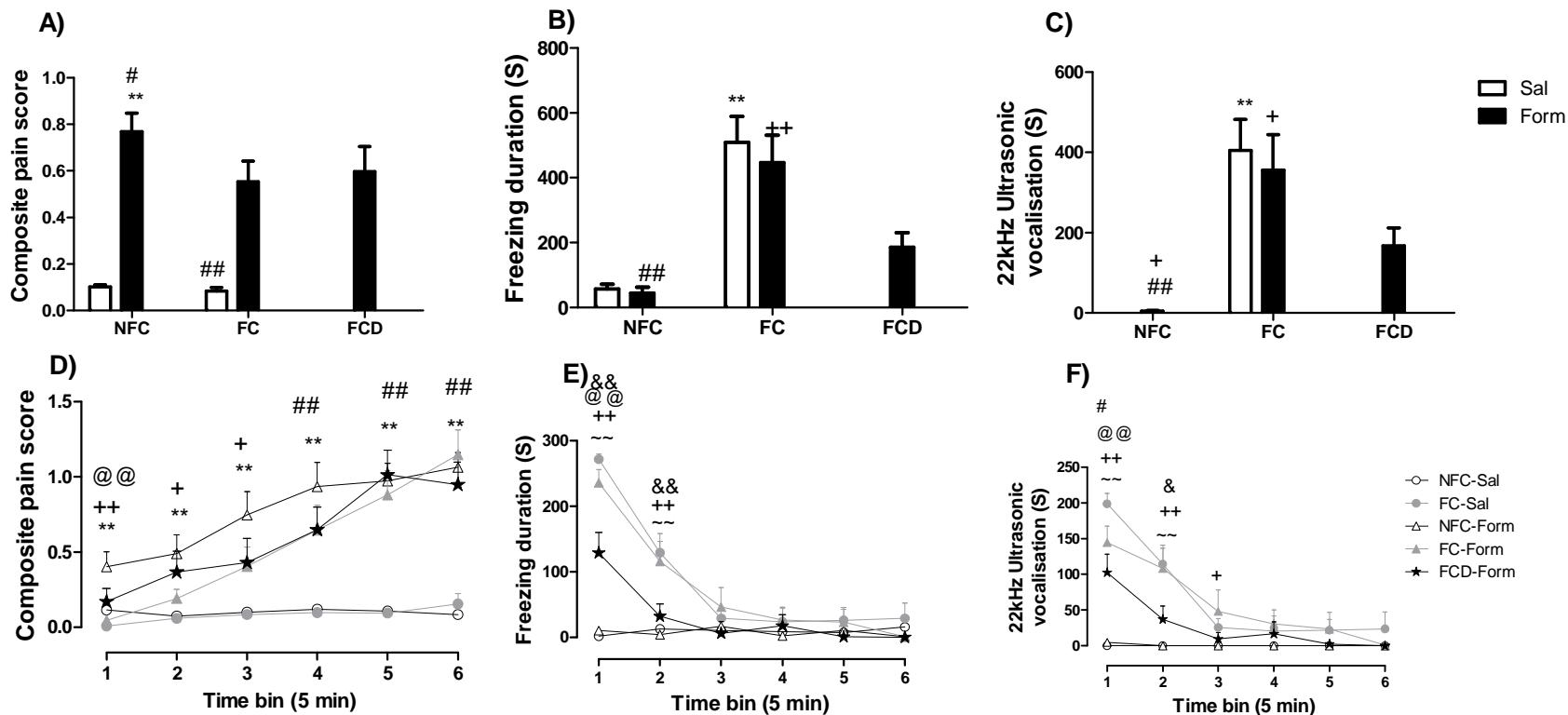
The SPSS 17.0 statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro-Wilk and Levene test, respectively. Behavioural and gene expression data were analysed using two-factor analysis of variance (ANOVA), with the factors being fear-conditioning and formalin (Experiment 1) or fear-conditioning and drug (Experiment 2). Time course behavioural data were analysed by repeated measures ANOVA with time as the within-subjects factor and group as the between-subjects factor. Post-hoc pairwise comparisons were made with Fisher's LSD when appropriate. Pearson coefficient was determined for correlation analysis. Data were expressed as group means  $\pm$  standard error of the mean ( $\pm$  SEM) and considered significant when  $P<0.05$ .

## **4.3 Results**

### **4.3.1 Experiment 1**

#### ***4.3.1.1 Effect of fear conditioning or/and intra-plantar formalin on nociceptive behaviour, expression of conditioned fear and FCA***

In non-fear conditioned rats, intra-plantar injection of formalin induced robust nociceptive behaviour as indicated by the significant increase in CPS (NFC-Sal vs. NFC-Form,  $p<0.01$  Fig. 4.1A&D). This increased nociceptive responding following intra-plantar injection of formalin was significantly attenuated in fear-conditioned rats when compared with rats that did not receive foot shock (NFC-Form vs. FC-Form,  $p<0.05$ , Fig 4.1A&D), confirming the expression of FCA. In fear-conditioned rats, intra-plantar injection of formalin only increased pain responding in the 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> 5 min time bin when compared with saline-injected controls (FC-Sal vs. FC-Form, Fig 4.1D). Contextual fear conditioning resulted in a significant increase in the duration of freezing and 22kHz ultrasonic vocalisation in saline- and formalin-injected rats re-exposed to the context compared to their respective non foot-shocked controls (NFC-Sal/Form vs. FC-Sal/Form,  $p<0.05$ , Fig. 4.1B&E). Furthermore, rats which were foot-shocked in a different context (FCD-Form) still showed increased duration of freezing and 22kHz ultrasonic vocalisation when compared with non foot-shocked counterparts (NFC-Form vs. FCD-Form,  $p<0.05$ ) but significantly lower than those foot-shocked in the same context (FCD-Form vs. FC-Form,  $p<0.05$ ) (Fig. 4.1B&C). Formalin-evoked nociceptive behaviour in FCD-Form rats was comparable with that in non foot-shocked controls, except for the first 5min bin where reduced nociceptive responding in the FCD-Form group was observed (NFC-Form vs. FCD-Form,  $p<0.01$ , Fig. 4.1D).



**Figure 4.1** Effects of fear-conditioning or intra-plantar formalin, alone or in combination, on (A) composite pain score for the total duration of the trial [ANOVA: fear conditioning  $F_{(2,55)}=1.56$ ,  $p=0.22$ ; formalin  $F_{(1,55)}=62.98$ ,  $p<0.001$ ; formalin x fear conditioning interaction  $F_{(1,55)}=1.91$ ,  $p=0.17$ ] B) the total duration of freezing [ANOVA: fear conditioning  $F_{(2,55)}=28.78$ ,  $p<0.01$ ; formalin  $F_{(1,55)}=0.41$ ,  $p=0.52$ ; formalin x fear conditioning interaction  $F_{(1,55)}=0.19$ ,  $p=0.67$ ] C) the total duration of 22kHz ultrasonic vocalisation [ANOVA: fear conditioning  $F_{(2,55)}=22.82$ ,  $p<0.01$ ; formalin  $F_{(1,55)}=0.16$ ,  $p=0.69$ ; formalin x fear conditioning interaction  $F_{(1,55)}=0.23$ ,  $p=0.63$ ] in rats during a 30min re-exposure to an observation chamber paired 24h previously with footshock. \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal; # $p<0.05$ , ## $p<0.01$  vs. FC-Form; + $p<0.05$ , ++ $p<0.01$  vs. FCD (Fisher's LSD post hoc test); D) CPS 5 min bin (repeated measure ANOVA: time  $F_{(5,275)}=24.5$ ,  $p<0.01$ , time x form  $F_{(5,275)}=14.5$ ,  $p<0.01$ ), E) duration of freezing 5min bin (repeated measure ANOVA: time  $F_{(5,275)}=56.9$ ,  $p<0.01$ , time x fear conditioning  $F_{(5,275)}=24.7$ ,  $p<0.01$ ) and F) duration of 22kHz ultrasonic vocalization 5 min bin (repeated measure ANOVA: time  $F_{(5,275)}=33.5$ ,  $p<0.01$ , time x fear conditioning  $F_{(5,275)}=12.4$ ,  $p<0.01$ ); \*\* $p<0.01$  NFC-Sal vs. NFC-Form, + $p<0.05$ , ++ $p<0.01$  NFC-Form vs. FC-Form, @@ $p<0.01$  NFC-Form vs. FCD-Form, # $p<0.05$ , ## $p<0.01$  FC-Sal vs. FC-Form, ~~~ $p<0.01$  NFC-Sal vs. FC-Sal, & $p<0.05$ , && $p<0.01$  FC-Form vs. FCD-Form; Data expressed as Mean  $\pm$  SEM ( $n=6$ ). FC, fear-conditioned; NFC, non-fear conditioned; FCD, fear-conditioned in a different context; Veh, vehicle

**4.3.1.2 Effects of formalin or/and fear conditioning on general exploratory/locomotor behaviours and hind paw oedema**

Both fear-conditioned and non fear-conditioned rats that received formalin showed significantly less general exploratory/locomotor behaviour as indicated by the sum of duration of walking, rearing and grooming (total activity) over the 30 min trial period, compared with saline-treated counterparts (NFC/FC-Sal vs. NFC/FC-Form,  $p<0.05$  Table 4.1). However, in both saline- and formalin-injected rats, fear conditioning did not affect total activity compared to respective non fear-conditioned controls (NFC-Sal/Form vs. FC- Sal/Form, Table 4.1). Intra-plantar injection of formalin produced significant oedema of the treated right hind paw in both fear-conditioned and non fear-conditioned rats as indicated by an increase in paw diameter following injection compared to saline-treated counterparts (NFC/FC-Sal vs. NFC/FC- Form ,  $p<0.01$ , Table 4.1).

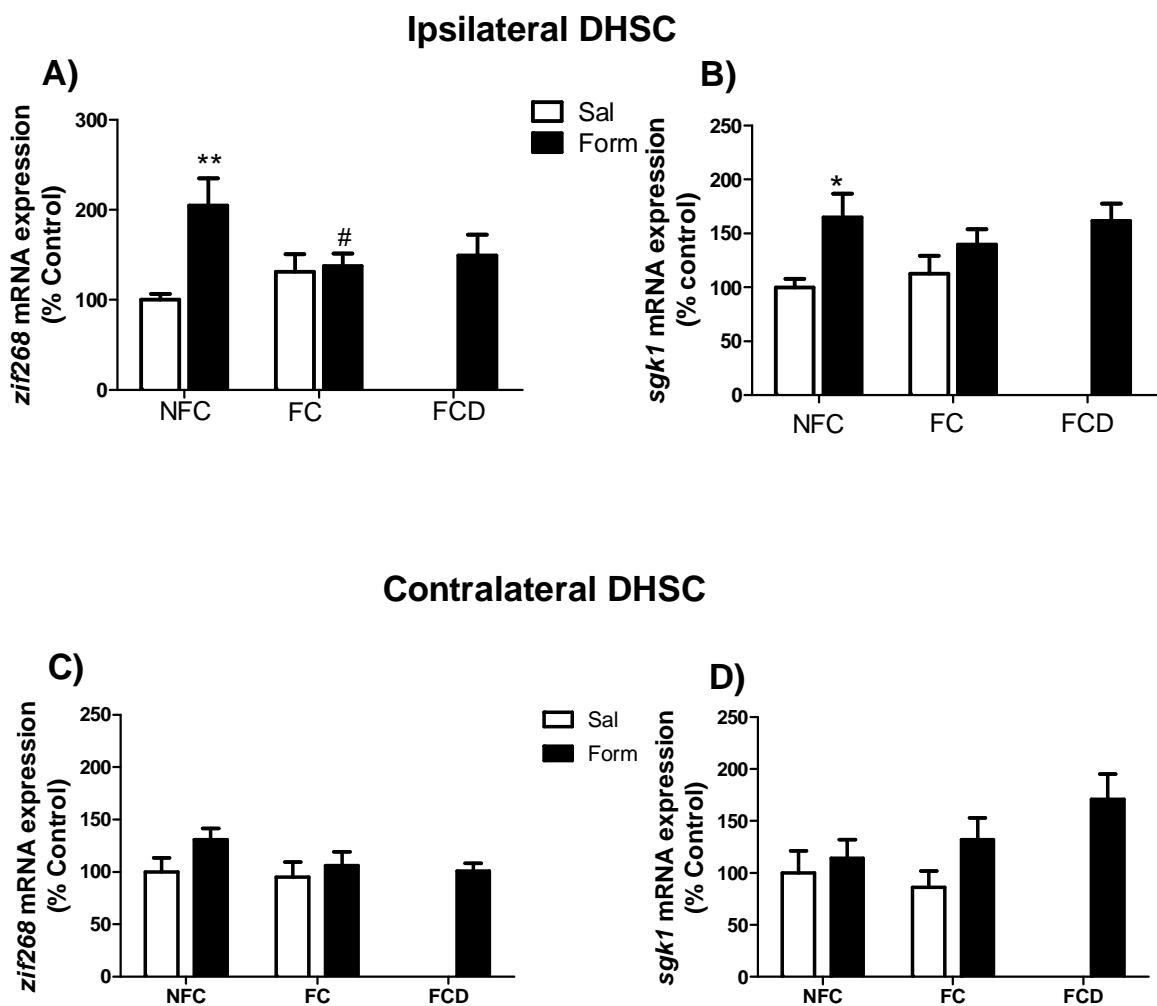
Groups	Change in paw diameter		Total activity	
	Mean(mm)	SEM	Mean(s)	SEM
NFC-Sal	0.20	$\pm 0.05$	517.17	$\pm 33.93$
NFC-Form	1.51	$\pm 0.08^{**}$	294.00	$\pm 44.75^*$
FC-Sal	0.21	$\pm 0.15$	452.83	$\pm 41.68$
FC-Form	1.32	$\pm 0.09^{++}$	236.58	$\pm 33.78^{++}$
FCD-Form	1.37	$\pm 0.10$	288.00	$\pm 34.05$

**Table 4.2** Effect of fear conditioning and intra-plantar formalin on paw diameter and locomotor activity; Effects of fear conditioning and intra-plantar formalin, alone or in combination, on paw diameter and total activity (measured as the sum of duration of rearing, grooming and walking) in rats during a 30 min re-exposure to an observation chamber which was paired with footshock 24h previously; Two-way ANOVA change in paw diameter (effect of fear conditioning  $F_{(2,55)}=0.44$ ,  $p=0.647$ , formalin  $F_{(1,55)}=153.36$ ,  $p<0.01$ , formalin x fear conditioning interaction  $F_{(1,55)}=1.528$ ,  $p=0.222$ ); total activity: (effect of fear conditioning  $F_{(2,55)}=1.41$ ,  $p=0.25$ , formalin  $F_{(1,55)}=33.56$ ,  $p<0.01$ , formalin x fear conditioning  $F_{(1,55)}=0.008$ ,  $p=0.93$ ). \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, ++ $p<0.05$  vs. FC-Sal; All data are expressed as Mean  $\pm$  SEM ( $n=12$ ). FC, fear-conditioned; NFC, non fear-conditioned; Sal, Saline; Form, Formalin

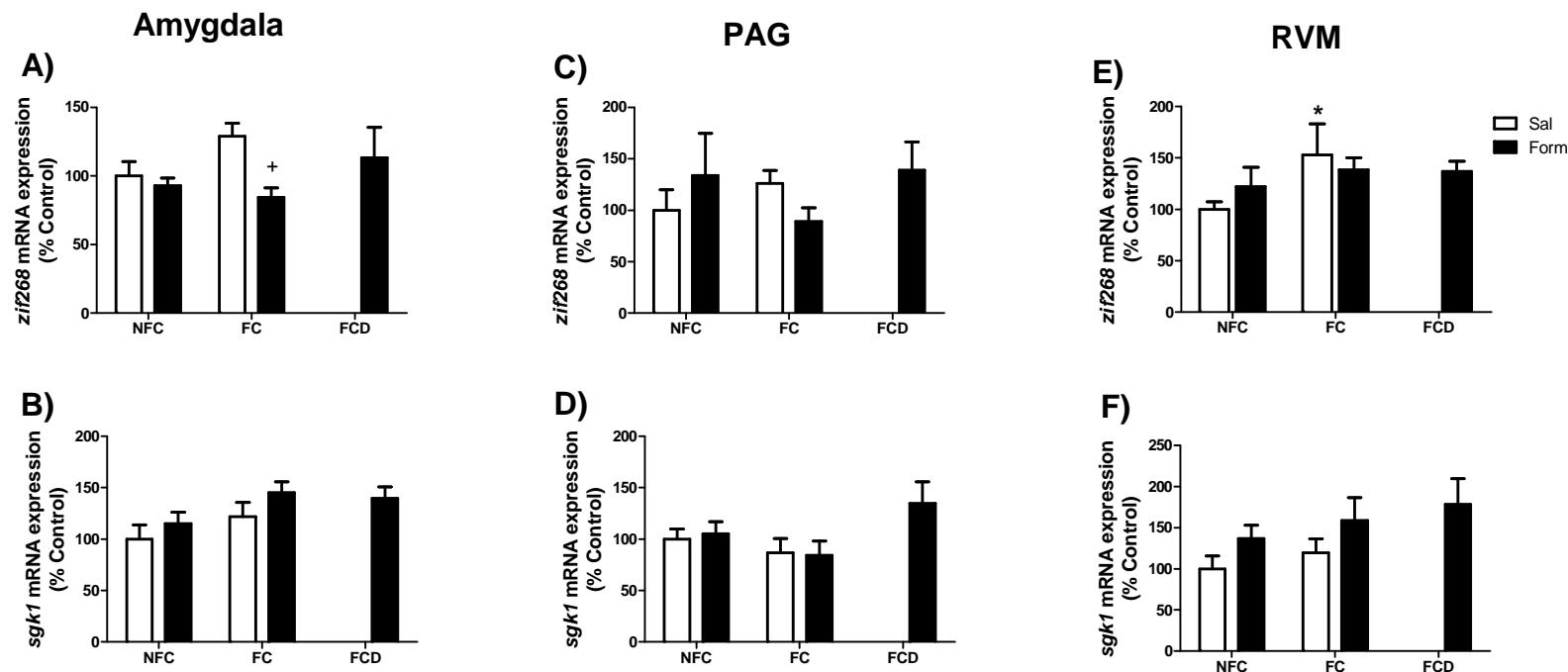
**4.3.1.3 Effect of fear conditioning, intra-plantar formalin and their combination on levels of expression of *Zif268* and *SGK1* in discrete brain regions and the spinal cord**

In non-fear conditioned rats, intra-plantar injection of formalin resulted in increased expression of *zif268* (Fig. 4.2A) and *sgk1* (Fig 4.2B) mRNA in the L4-L6 segment of the ipsilateral DHSC (NFC-Sal vs. NFC-Form,  $p<0.05$ ) without affecting levels in the

contralateral DHSC, amygdala, PAG and RVM (Fig 4.2(C&D) & 4.3), compared with saline-treated counterparts. Compared with non fear-conditioned saline-treated rats, fear-conditioned rats that received intra-plantar saline injection showed increased expression of *zif268* mRNA in the RVM (NFC-Sal vs. FC- Sal, p<0.05, Fig. 4.3E) and showed a strong trend towards increased expression of *zif268* mRNA in the amygdala (Fig 4.3A) but no change in levels in the ipsilateral (Fig 4.2A) and contralateral DHSC (Fig 4.2C) or the PAG (Fig 4.3C). Fear-conditioned rats that received intra-plantar formalin (i.e. those expressing FCA) showed decreased expression of *zif268* mRNA and a trend towards decreased expression of *sgk1* mRNA in the ipsilateral DHSC when compared with non fear-conditioned rats that received formalin (NFC-Form vs. FC-Form, Fig. 4.2A&B). However, formalin-injected, fear-conditioned rats did not show any alterations in the expression of *zif268* and *sgk1* mRNA in the contralateral DHSC (Fig 4.2C&D) or the amygdala, PAG, and RVM (Fig 4.3), compared with non fear-conditioned counterparts. In fear-conditioned rats, intra-plantar formalin decreased expression of *zif268* mRNA in the amygdala (FC-Sal vs. FC-Form, p<0.05, Fig 4.3A) and tended to increase *sgk1* mRNA expression in the contralateral DHSC (Fig 4.2D) compared with saline-injected counterparts without affecting levels of expression in the PAG, RVM (Fig 4.3) or ipsilateral DHSC (Fig 4.2B). In addition, in rats which were footshocked in a different context (FCD-Form) there were no significant changes in the level of expression of *zif268* and *sgk1* mRNA in the amygdala, RVM and DHSC, compared with rats which were footshocked and re-exposed to the same context (FC-Form vs. FCD-Form, Fig 4.2 &4.3). However, a strong trend towards increased expression of both *zif268* and *sgk1* mRNA in the PAG (Fig 4.3C&D) and increased expression of *sgk1* mRNA in the contralateral DHSC (Fig 4.2D) were seen in FCD-Form rats compared with rats fear-conditioned in the same context (FC-Form vs. FCD-Form). FCD-Form rats also showed a trend to decreased expression of *zif268* mRNA in the ipsilateral DHSC when compared with non fear-conditioned formalin-injected rats (NFC-Form vs. FCD-Form, Fig 4.2A). The levels of  $\beta$ -actin and GAPDH mRNA, which were used as endogenous control/reference genes, were not affected by any of the treatments (data not shown).



**Figure 4.2** Effects of fear-conditioning and intra-plantar formalin, alone or in combination, on levels of expression of *zif268* and *sgk1* mRNA in the ipsilateral and contralateral DHSC; A) *zif268* mRNA expression in the ipsilateral DHSC (ANOVA: formalin:  $F_{(1, 37)}=7.31$ ,  $p<0.05$ ; fear-conditioning:  $F_{(2,37)}=0.76$ ,  $p=0.48$  and formalin x fear-conditioning interaction:  $F_{(1,37)}=5.69$ ,  $p<0.05$ ) B) *sgk1* mRNA expression in the ipsilateral DHSC (ANOVA: formalin:  $F_{(1, 37)}=7.87$ ,  $p<0.01$ ; fear-conditioning:  $F_{(2,37)}=0.18$ ,  $p=0.83$  and formalin x fear-conditioning interaction:  $F_{(1,37)}=1.37$ ,  $p=0.25$ ) C) *zif268* mRNA in the contralateral DHSC (ANOVA: formalin:  $F_{(1, 25)}=2.95$ ,  $p=0.09$ ; fear-conditioning:  $F_{(2,25)}=1.46$ ,  $p=0.252$  and formalin x fear-conditioning interaction:  $F_{(1,25)}=0.66$ ,  $p=0.42$ ) D) *sgk1* mRNA in the contralateral DHSC (ANOVA: formalin:  $F_{(1, 25)}=2.21$ ,  $p=0.15$ ; fear-conditioning:  $F_{(2,25)}=1.82$ ,  $p=0.18$  and formalin x fear-conditioning interaction:  $F_{(1,25)}=0.62$ ,  $p=0.44$ ). \* $p<0.05$ , \*\* $p<0.01$  vs. NFC-Sal, # $p<0.05$ , vs. NFC-Form (Fisher's LSD post hoc test); Data expressed as Mean  $\pm$  SEM as a percentage of the control (NFC-Sal) group ( $n=5-6$ ). FC, fear-conditioned; FCD, fear-conditioned in a different context; NFC, non-fear conditioned; Sal, Saline; Form, Formalin.

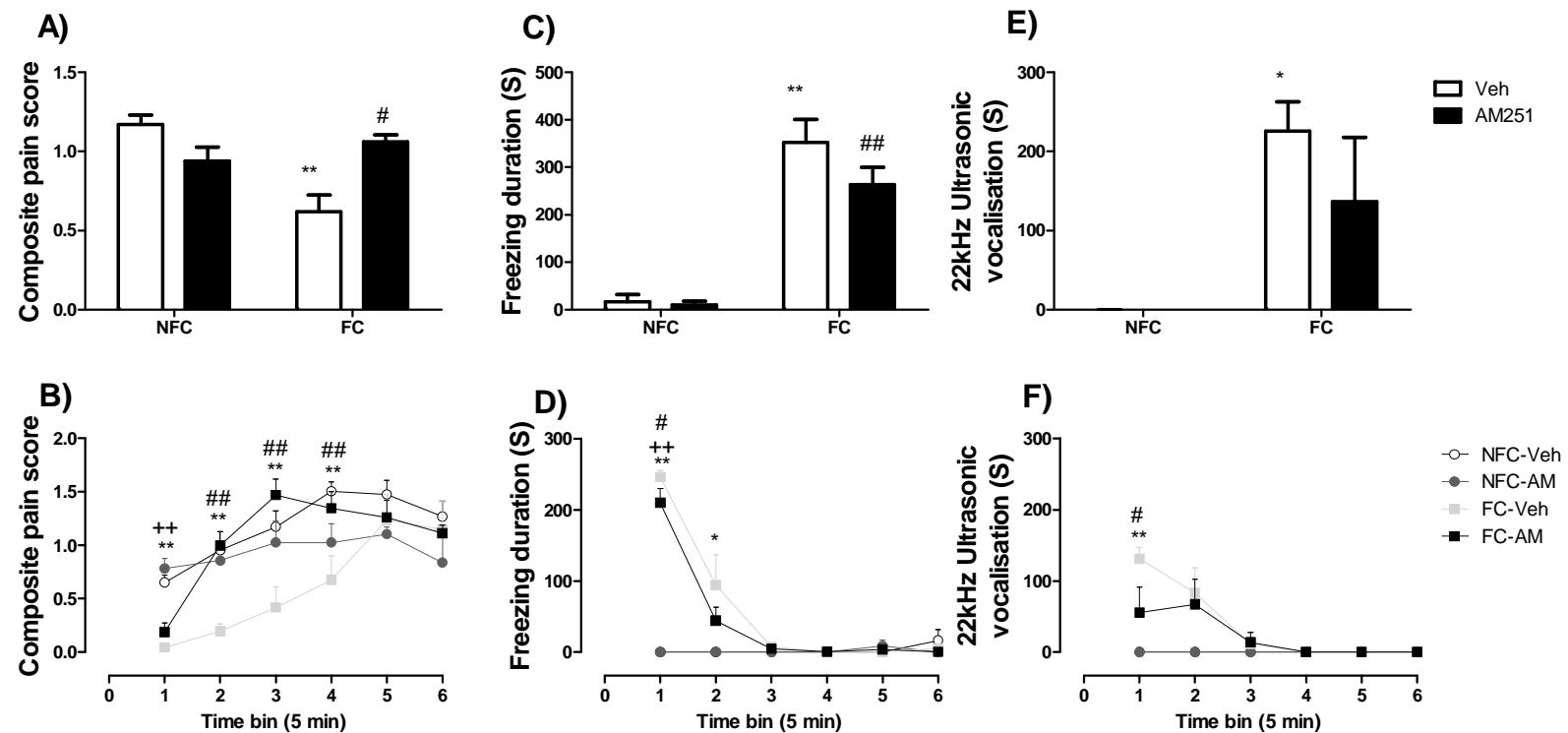


**Figure 4.3** Effects of fear-conditioning and intra-plantar formalin, alone or in combination, on levels of expression of *zif268* and *sgk1* mRNA in the amygdala, PAG and RVM; A) *zif268* mRNA expression in the amygdala (ANOVA: formalin:  $F_{(1, 25)}=4.3$ ,  $p<0.05$ ; fear-conditioning:  $F_{(2,25)}=0.15$ ,  $p=0.21$  and formalin x fear-conditioning interaction:  $F_{(1,25)}=2.25$ ,  $p=0.146$ ) B) *sgk1* mRNA expression in the amygdala (ANOVA: formalin:  $F_{(1, 25)}=2.5$ ,  $p=0.13$ ; fear-conditioning:  $F_{(2,25)}=2.44$ ,  $p=0.11$  and formalin x fear-conditioning interaction:  $F_{(1,25)}=11$ ,  $p=0.74$ ) C) *zif268* mRNA in the PAG (ANOVA: formalin:  $F_{(1, 23)}=0.004$ ,  $p=0.95$ ; fear-conditioning:  $F_{(1,23)}=0.55$ ,  $p=0.59$  and formalin x fear-conditioning interaction:  $F_{(1,23)}=0.69$ ,  $p=0.16$ ) D) *sgk1* mRNA in the PAG (ANOVA: formalin:  $F_{(1, 23)}=0.01$ ,  $p=0.93$ ; fear-conditioning:  $F_{(2,23)}=3.1$ ,  $p=0.063$  and formalin x fear-conditioning interaction:  $F_{(1,23)}=0.07$ ,  $p=0.79$ ) E) *zif268* mRNA in the RVM (ANOVA: formalin:  $F_{(1, 24)}=0.05$ ,  $p=0.81$ ; fear-conditioning:  $F_{(2,24)}=3.9$ ,  $p=0.046$  and formalin x fear-conditioning interaction:  $F_{(1,24)}=1.23$ ,  $p=0.28$ ) F) *sgk1* mRNA in the RVM (ANOVA: formalin:  $F_{(1, 25)}=2.85$ ,  $p=0.10$ ; fear-conditioning:  $F_{(1,25)}=1.01$ ,  $p=0.37$  and formalin x fear-conditioning interaction:  $F_{(1,25)}=0.00$ ,  $p=0.97$ ) \* $p<0.05$  vs. NFC-Sal, + $p<0.05$ , vs. FC-Sal (Fisher's LSD post hoc test); Data expressed as Mean  $\pm$  SEM as a percentage of the control (NFC-Sal) group ( $n=5-6$ ). FC, fear-conditioned; FCD, fear-conditioned in a different context; NFC, non-fear conditioned; Sal, Saline; Form, Formalin

### **4.3.2 Experiment 2**

#### **4.3.2.1 Effect of systemic administration of AM251 on FCA, pain- and fear-related behaviours**

In non-fear conditioned rats, intra-plantar injection of formalin significantly induced robust nociceptive behaviour as indicated by the CPS (Fig.4.4A & B). This formalin-evoked nociceptive responding in non fear-conditioned rats was not affected by systemic administration of AM251 although there was a trend towards decreased nociceptive behaviour in the AM251-treated group compared with vehicle-treated counterparts (NFC-Veh vs. NFC-AM251,  $p=0.054$ ). Formalin-induced nociceptive behaviour was significantly reduced in fear-conditioned rats compared with non fear-conditioned counterparts, confirming the expression of FCA (NFC-Form vs. FC-Form,  $p<0.05$ ). Systemic administration of AM251 to fear-conditioned rats significantly increased nociceptive behaviour when compared to vehicle-treated counterparts, resulting in near complete attenuation of FCA (FC-Veh vs. FC-AM251,  $p<0.05$ ). Non fear-conditioned rats showed almost no fear-related behaviour as measured by the duration of freezing and 22kHz ultrasonic vocalisation. However, contextual fear-conditioning resulted in a significant increase in the duration of freezing and 22kHz ultrasonic vocalisation in formalin-injected rats re-exposed to the context previously associated with footshock compared to their respective non fear-conditioned controls (NFC-Veh vs. FC-Veh,  $p<0.05$ , Fig 4.4C-F). Furthermore, in fear-conditioned rats, systemic administration of AM251 significantly reduced the duration of freezing behaviour and duration of 22kHz ultrasonic vocalisation over the first 5 minutes of the trial when compared with vehicle-treated controls (FC-Veh vs. FC-AM251,  $p<0.05$ , Fig 4.4C-F). In AM251-treated rats, fear conditioning significantly increased duration of freezing but did not affect duration of 22kHz ultrasonic vocalisation (NFC-AM251 vs. FC-AM251).



**Figure 4.4** Effects of fear-conditioning and intra-peritoneal administration of AM251 (3mg/kg) on (A) the composite pain score for the entire 30 min trial (ANOVA: drug  $F_{(1,19)}=1.83$ ,  $p=0.19$ ; fear-conditioning  $F_{(1,19)}=7.54$ ,  $p<0.05$ ; drug x fear conditioning interaction  $F_{(1,19)}=18.7$ ,  $p<0.01$ ) (B) the composite pain score as 5 min bins (repeated measure ANOVA: time  $F_{(5,95)}=18.2$ ,  $p<0.01$ , time x fear conditioning  $F_{(5,95)}=2.59$ ,  $p<0.05$ , time x drug  $F_{(5,95)}=3.50$ ,  $p<0.01$ , time x drug x fear conditioning  $F_{(5,95)}=2.78$ ,  $p<0.05$ ), (C) total duration of freezing over the entire 30 min trial (ANOVA: drug  $F_{(1,19)}=2.09$ ,  $p=16$ ; fear-conditioning  $F_{(1,19)}=79.45$ ,  $p<0.01$ ; drug x fear conditioning interaction  $F_{(1,19)}=1.52$ ,  $p=0.233$ ) (D) duration of freezing as 5min bins (repeated measure ANOVA: time  $F_{(5,95)}=61.1$ ,  $p<0.01$ , time x fear conditioning  $F_{(5,95)}=65.00$ ,  $p<0.01$ ) (E) total duration of 22 kHz ultrasonic vocalisation over the entire 30 min trial (ANOVA: drug  $F_{(1,19)}=0.89$ ,  $p=0.35$ ; fear-conditioning  $F_{(1,19)}=14.62$ ,  $p<0.01$ ; drug x fear conditioning interaction  $F_{(1,19)}=0.89$ ,  $p=0.36$ ) (F) duration of 22 kHz ultrasonic vocalisation as 5 min bins (repeated measure ANOVA: time  $F_{(5,95)}=11.09$ ,  $p<0.01$ , time x fear conditioning  $F_{(5,95)}=11.08$ ,  $p<0.01$ ) in formalin-injected rats during the 30min re-exposure to an observation chamber paired 24h previously with footshock. \*  $p<0.05$ , \*\*  $p<0.01$  NFC-Veh vs. FC-Veh; + $p<0.05$ , ++ $p<0.01$  NFC-AM251 vs. FC-AM251; # $p<0.05$ , ## $p<0.01$  FC-Veh vs. FC-AM251 (Fisher's LSD post hoc test); Data expressed as Mean  $\pm$  SEM ( $n=6$ ). FC, fear-conditioned; NFC, non-fear conditioned; Veh, vehicle

**4.3.2.2 Effects of AM251 and fear conditioning on general exploratory/locomotor behaviours, defecation and hind paw oedema**

Intra-plantar injection of formalin produced comparable oedema of the treated right hind paw in both fear-conditioned and non fear-conditioned rats as indicated by an increase in paw diameter (NFC- Form vs. FC-Form, Table 4.2). Intra-peritoneal AM251 did not affect the changes in paw diameter in either fear-conditioned or non fear-conditioned rats compared to vehicle treated counterparts (NFC/FC-Veh vs. NFC/FC-AM251, Table 4.2). In addition, comparison between fear-conditioned and non fear-conditioned rats that received AM251 did not show a difference in change in their paw diameter following formalin injection (NFC-AM251 vs. FC-AM251, Table 4.2). Neither fear conditioning nor AM251 significantly affected general exploratory/locomotor behaviour as indicated by the sum of duration of walking, rearing and grooming (total activity) over the 30 min trial period. However, in fear-conditioned rats, systemic AM251 showed a strong trend to decrease total activity when compared with vehicle treated controls (FC-Veh vs. FC-AM251, Table 4.2). In both vehicle- and AM251-treated rats, fear-conditioning significantly increased defecation compared to respective non fear-conditioned controls (NFC-Veh/AM251 vs. FC-Veh/AM251, p<0.05 Table 4.2). However, in both fear-conditioned and non fear-conditioned rats, no effect of AM251 was seen on defecation compared to respective vehicle-treated rats (NFC/FC -Veh vs. NFC/FC -AM251, Table 4.2).

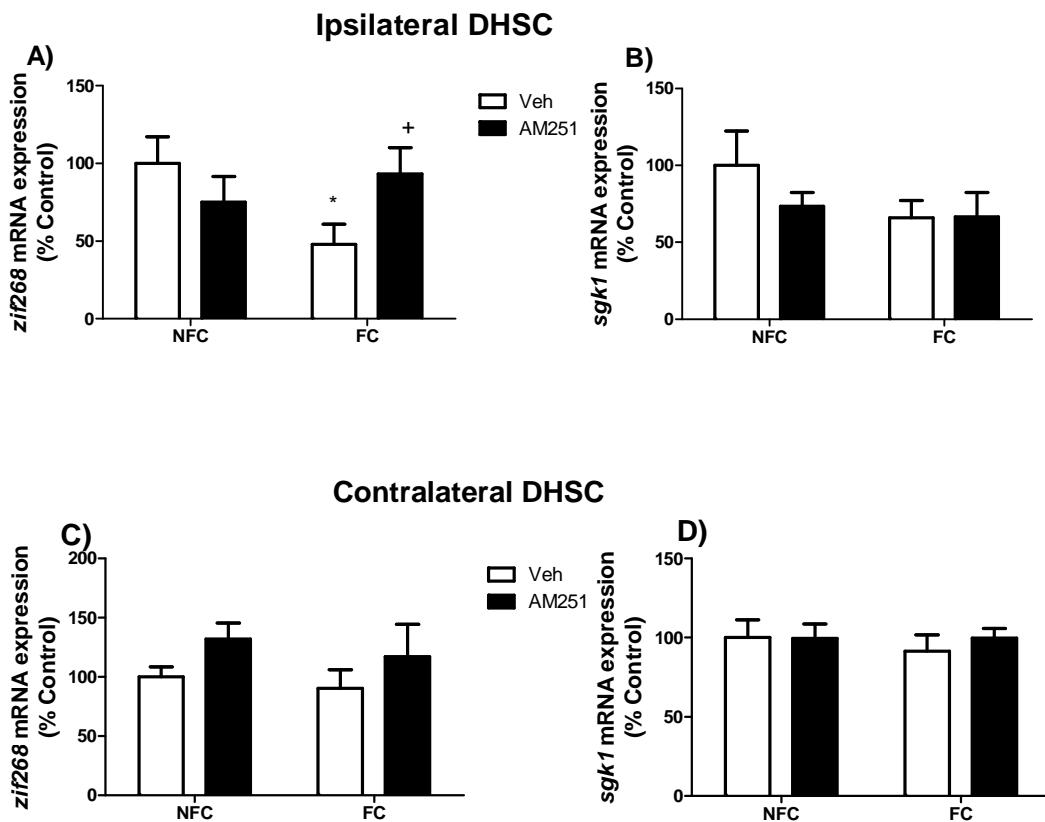
Groups	Change in paw diameter (mm)		Defecation (number of pellets)		Total activity (s)	
	Mean	±SEM	Mean	±SEM	Mean	±SEM
NFC-Veh	2.09	±0.08	3.75	±0.45	182.39	±21.17
NFC-AM251	1.81	±0.13	3.43	±0.75	178.45	±38.14
FC-Veh	1.91	±0.06	5.88	±0.44**	189.52	±25.73
FC-AM251	1.91	±0.10	5.14	±0.40 <sup>+</sup>	113.68	±17.54

**Table 4.3** Effect of AM251 and fear conditioning on paw diameter, defecation and locomotor activity in formalin-injected rats; Effects of AM251 and fear conditioning, alone or in combination, on paw diameter and total activity (measured as the sum of duration of rearing, grooming and walking) in formalin-injected rats during a 30 min re-exposure to an observation chamber which was paired with footshock 24h previously. Two-way ANOVA: change in paw diameter (effect of fear conditioning  $F_{(1,27)}=0.16$ ,  $p=0.69$ , drug  $F_{(1,27)}=2.05$ ,  $p=0.16$ , drug x fear conditioning interaction  $F_{(1,27)}=2.05$ ,  $p=0.16$ ); Defecation (effect of fear conditioning  $F_{(1,26)}=13.51$ ,  $p=<0.01$ , drug  $F_{(1,26)}=1.02$ ,  $p=0.32$ , drug x fear conditioning interaction  $F_{(1,26)}=0.15$ ,  $p=0.69$ ); total activity: (effect of fear conditioning  $F_{(1,19)}=1.26$ ,  $p=0.27$ , drug  $F_{(1,19)}=2.41$ ,  $p=0.14$ , drug x fear conditioning interaction  $F_{(1,19)}=1.96$ ,  $p=0.18$ ). \*\* $p<0.05$  vs. NFC-Veh, + $p<0.05$  vs. FC-Veh; All data are expressed as Mean ± SEM (n=12). FC, fear-conditioned; NFC, non fear-conditioned; Veh, vehicle

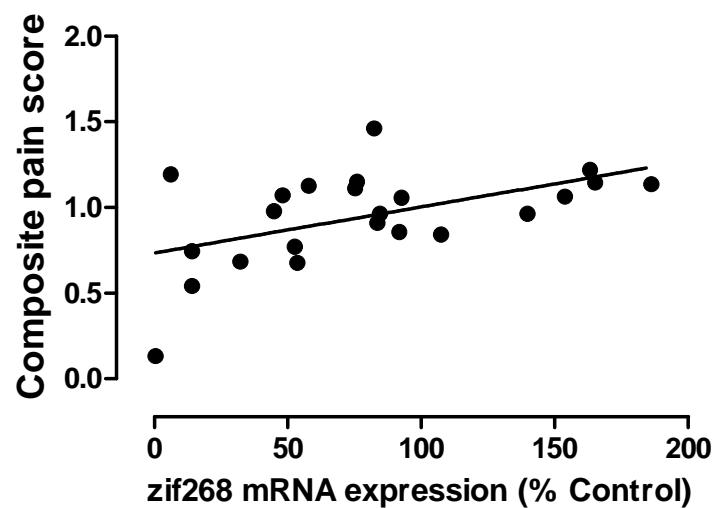
#### **4.3.2.3 Effect of fear conditioning and systemic administration of AM251 on expression of *zif268* and *sgk1* mRNA in the DHSC in formalin-injected rats**

In vehicle-treated rats, fear conditioning significantly decreased the expression of *zif268* mRNA in the ipsilateral DHSC (NFC-Veh vs. FC-Veh,  $p<0.05$ , Fig 4.5A) without affecting levels in the contralateral DHSC (Fig 4.5C), compared with non fear-conditioned formalin-injected counterparts. Thus, FCA was associated with a suppression of *zif268* mRNA expression in the ipsilateral DHSC. However, fear conditioning did not significantly affect expression of *sgk1* mRNA levels in the ipsilateral or contralateral DHSC of vehicle-treated rats, although there was a trend towards decreased levels in the ipsilateral side compared to non fear-conditioned controls (NFC-Veh vs. FC-Veh, Fig 4.5B). In non fear-conditioned rats, systemic administration of AM251 did not affect expression of either *zif268* or *sgk1* mRNA in either side of the DHSC when compared with vehicle-treated controls, though a trend towards increased expression of *zif268* mRNA was seen in the contralateral DHSC (NFC-Veh vs. NFC-AM251, Fig 4.5). In fear-conditioned rats, AM251 significantly increased the expression of *zif268* mRNA in the ipsilateral DHSC (FC-Veh vs. FC-AM251,  $p<0.05$ , Fig 4.5A) without affecting levels of expression of *zif268*

mRNA in the contralateral DHSC or *sgk1* mRNA in either side (FC-Veh vs. FC-AM251, Fig 4.5). Thus, systemic administration of AM251 significantly attenuated the conditioned fear-induced suppression of *zif268* mRNA expression in the ipsilateral DHSC. Levels of *GAPDH* mRNA, which was used as an endogenous control, were not affected by any of the treatments (data not shown). Nociceptive behaviour (CPS) in formalin-treated rats correlated positively with *zif268* expression in the ipsilateral DHSC ( $r=0.52$ ,  $p<0.05$ , Fig. 4.6).



**Figure 4.5** Effects of fear-conditioning or systemic administration of AM251 (3mg/kg, i.p.) in the presence of intra-plantar formalin on levels of expression of *zif268* and *sgk1* mRNA in the ipsilateral and contralateral DHSC; A) *zif268* mRNA expression in the ipsilateral DHSC (ANOVA: drug:  $F_{(1, 28)}=0.41$ ,  $p=0.53$ ; fear conditioning:  $F_{(1, 28)}=1.15$ ,  $p=0.29$  and formalin x fear-conditioning interaction:  $F_{(1, 28)}=4.89$ ,  $p<0.05$ ) B) *sgk1* mRNA expression in the ipsilateral DHSC (ANOVA: drug:  $F_{(1, 28)}=0.77$ ,  $p=387$ ; fear conditioning:  $F_{(1, 28)}=1.3$ ,  $p=0.26$  and formalin x fear conditioning interaction:  $F_{(1, 28)}=0.84$ ,  $p=0.36$ ) (C) *zif268* mRNA expression in the contralateral DHSC (ANOVA: drug:  $F_{(1, 28)}=2.7$ ,  $p=0.11$ ; fear conditioning:  $F_{(1, 28)}=0.48$ ,  $p=0.49$  and formalin x fear conditioning interaction:  $F_{(1, 28)}=0.02$ ,  $p=0.88$ ) (D) *sgk1* mRNA expression in the contralateral DHSC (ANOVA: drug:  $F_{(1, 28)}=0.17$ ,  $p=0.68$ ; fear conditioning:  $F_{(1, 28)}=0.19$ ,  $p=0.66$  and formalin x fear conditioning interaction:  $F_{(1, 28)}=0.2$ ,  $p=0.65$ ). \* $p<0.05$  vs. NFC-Veh, + $p<0.05$  vs. FC-Veh (Fisher's LSD post hoc test); Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). FC, fear-conditioned; NFC, non-fear conditioned; Sal, Saline; Form, Formalin



**Figure 4.6** Correlation between the composite pain score (CPS) and the expression of *zif268* mRNA in the ipsilateral DHSC in formalin-treated rats (Pearson correlation coefficient  $r=0.52$ ;  $r^2=0.27$ ,  $p=0.011$ .

#### **4.4 Discussion**

The present study demonstrated that formalin-evoked nociceptive behaviour is associated with increased expression of the plasticity-related genes *zif268* and *sgk1* in the ipsilateral DHSC. This effect was attenuated in fear-conditioned rats that expressed fear-induced suppression of formalin-evoked nociceptive behaviour (i.e. FCA). Contextual fear conditioning resulted in increased expression of *zif268* in the RVM and showed a strong trend towards increased expression in the amygdala which was reversed in the presence of nociceptive tone. In addition, systemic administration of the CB<sub>1</sub> receptor antagonist/inverse agonist AM251 prevented FCA and reduced fear-related behaviour in the presence of nociceptive tone. In addition, AM251 reversed the attenuation of *zif268* expression in the ipsilateral DHSC that was associated with FCA. Together, these results suggest that *zif268* expression in the DHSC is an important molecular correlate of endocannabinoid-mediated FCA.

Previously, increased expression of the plasticity-related genes *zif268* and *sgk1* in the DHSC was demonstrated in response to noxious stimuli (Delander *et al.*, 1997; Geranton *et al.*, 2007; Otahara *et al.*, 2003; Rahman *et al.*, 2002). *zif268* is rapidly induced in dorsal horn neurons within 30min of noxious stimulation (Wisden *et al.*, 1990) and is often studied as a marker of neuronal activity. A role for *zif268* in the maintenance of inflammatory pain has been demonstrated following infusion of antisense *zif268* and in *zif268* knockout mice (Ko *et al.*, 2005) where nociceptive response to peripheral inflammation was significantly reduced. Spinal LTP is also correlated with increased neuronal expression of *zif268* in the superficial dorsal horn and that intra-thecal *zif268* antisense treatment resulted in decreased inflammatory hyperalgesia (Rygh *et al.*, 2006). Similarly, *sgk1* expression has been found to be increased in the DHSC in the rat complete Freund's adjuvant model of chronic inflammatory pain (Geranton *et al.*, 2007). Intrathecal injection of antisense oligonucleotides directed against *sgk1* resulted in a delay in the onset of pain-related behaviour for at least 24h, suggesting a role for *sgk1* in the induction of inflammatory pain states (Geranton *et al.*, 2007). In line with these findings, in the present study, formalin-evoked nociceptive behaviour was accompanied by increased expression of both

*zif268* and *sgk1* in the ipsilateral DHSC with no changes observed in the contralateral side. In addition, we demonstrated a positive correlation between DHSC expression of *zif268* and formalin-induced nociception. We observed a reduction in nociceptive behaviour upon re-exposure to a context previously paired with footshock confirming previous reports of the phenomenon of FCA (Butler et al., 2008, 2011; Finn et al., 2004, 2006; Helmstetter and Fanselow, 1987; Roche et al., 2007). This potent suppression of pain-related behaviour was accompanied by attenuation of the formalin-evoked increase in the expression of *zif268* in the ipsilateral DHSC (and a strong trend in a similar direction for *sgk1*). These data suggest that fear-induced activation of the descending inhibitory pain pathway may result in suppression of pain-evoked *zif268* expression within the DHSC.

To determine the involvement of the CB<sub>1</sub> receptor in this fear-induced suppression of pain-evoked *zif268* expression, we investigated the expression of *zif268* in the DHSC following systemic administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, AM251, in fear-conditioned, formalin-treated rats (experiment 2). Again, robust FCA was observed and was associated with suppression of *zif268* (and strong trend in a similar direction for *sgk1*) in the ipsilateral DHSC in a similar manner to experiment 1. In addition, systemic administration of AM251 attenuated FCA and prevented the fear-induced reduction of *zif268* expression in formalin-treated rats. AM251 had no effect on either formalin-evoked nociceptive behaviour or on expression of *zif268* in non fear-conditioned rats, suggesting a specific effect on FCA and associated alteration in DHSC *zif268* expression. The attenuation of FCA by systemic AM251 is in line with previous report showing that systemic administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, attenuated FCA in rats (Finn et al., 2004). As AM251 did not have any effect on nociceptive behaviour *per se* or locomotor activity, this suggests a specific effect of AM251 on FCA and confirms that the FCA expressed during this experiment is CB<sub>1</sub>-dependent and probably endocannabinoid-mediated. To date, the molecular correlates of endocannabinoid-mediated FCA have not been examined in detail. The present findings indicate that *zif268* expression in the DHSC could be an important

molecular mediator of this endocannabinoid-mediated FCA. Previous work showed that the DHSC is important for endocannabinoid-mediated suppression of pain responding following exposure to unconditioned stress (Suplita Li *et al.*, 2006). Our findings here support and extend this finding by suggesting that the DHSC is also a relevant structure in endocannabinoid-mediated suppression of pain responding induced by psychological, conditioned stress/fear. Several lines of evidence suggest that cannabinoid CB<sub>1</sub> receptor activation induces the expression of *zif268* *in vitro* (Bouaboula *et al.*, 1995a; Graham *et al.*, 2006; Valjent *et al.*, 2001) and *in vivo* (Mailleux *et al.*, 1994). However, our data here suggest that CB<sub>1</sub> blockade can attenuate the suppression of *zif268* expression that accompanies FCA. It is possible that in the presence of nociceptive stimuli and/or fear, as was the case in this experiment, the effect of endocannabinoid-induced stimulation of the CB<sub>1</sub> receptor on *zif268* may be different to the effects of potent CB<sub>1</sub> receptor agonists under different physiological conditions. To date, there have been no studies investigating the effects of CB<sub>1</sub> receptor activation on *sgk1* expression. However, the modest, non-significant decrease in *sgk1* expression observed here in the ipsilateral DHSC in association with FCA may be non-CB<sub>1</sub> receptor mediated as it was not prevented by AM251 under the present experimental conditions. Finally, if an increase in *zif268* mRNA expression reflects increased neuronal activity, then the present findings suggest that endocannabinoid-mediated FCA involves suppression of neuronal activity in the DHSC.

Intra-plantar formalin did not affect expression of either *zif268* or *sgk1* in the amygdala, PAG or RVM. It has been recently demonstrated in a rat model of neuropathic pain with chronic constriction injury that *zif268*-immunoreactivity is enhanced in the thalamus and PAG but unaltered in the amygdala (Pagano *et al.*, 2011). *zif268* expression has been shown to be increased in the hippocampus and anterior cingulate cortex following amputation injury (Wei *et al.*, 1999; Wei *et al.*, 2000). In addition, fos and Erk, other markers of neuronal activity, have been shown to increase in the RVM following formalin injection into the hind paw (Imbe *et al.*, 2008; Oh *et al.*, 2006). To date, altered *sgk1* expression following noxious stimuli has only been reported in the DHSC (Geranton *et al.*,

2007). Although our studies suggest a lack of effect of intraplantar formalin injection on *zif268* or *sgk1* expression in supraspinal areas, it is possible that, alterations in these genes may occur at a time point other than those examined here in other brain regions, or following a different form of noxious stimulus. Indeed, Geranton *et al.*, showed that temporal patterns of gene expression with distinct pattern of gene expression at each of the time points follow the initial inflammatory stimulus following CFA injection in the ankle joint (Geranton *et al.*, 2007).

Both the RVM (Vianna *et al.*, 2008) and amygdala (Davis *et al.*, 1999; LeDoux, 2000; Swanson *et al.*, 1998; Weidenfeld *et al.*, 2002) are important neural structures in the expression of conditioned-fear. In the current experiment, conditioned-fear was associated with increased expression of *zif268* in the RVM with a strong similar trend in the amygdala. These findings support a large body of previous work demonstrating enhanced *zif268* expression during fear/anxiety in the hippocampus (Hall *et al.*, 2001; Lonergan *et al.*, 2010) and amygdala (Busti *et al.*, 2011; Hall *et al.*, 2000; Hall *et al.*, 2001; Malkani *et al.*, 2000; Perez-Villalba *et al.*, 2008; Ressler *et al.*, 2002; Rosen *et al.*, 1998a) Moreover, expression of *zif268* in the amygdala has been shown to be functionally important in contextual fear memory consolidation (Malkani *et al.*, 2004). Although the RVM is involved in the cardiovascular and somatic components of conditioned-fear (Vianna *et al.*, 2008), to our knowledge, this is the first report of increased expression of *zif268* in the RVM during conditioned-fear. However, restraint stress has been shown to activate Erk, another marker of neuronal activation, in the RVM of rats (Imbe *et al.*, 2004). In addition, unlike non-fear-conditioned rats, in fear-conditioned rats, intra-plantar formalin decreased the expression of *zif268* in the amygdala, suggesting a differential response of *zif268* to noxious stimuli in the presence versus absence of fear in this brain region. Despite the key role of the PAG in aversion and fear (Amorapanth *et al.*, 1999; Bandler *et al.*, 1985; Carrive *et al.*, 1999; Carrive *et al.*, 1997; Krieger *et al.*, 1985; LeDoux, 1998; Schenberg *et al.*, 1990), expression of *zif268* was not altered in this brain region during expression of conditioned fear under the present experimental conditions. In contrast to *zif268*, no alteration in expression

of *sgk1* was seen in the amygdala, PAG or RVM during conditioned-fear. Again to our knowledge this is the first study to investigate changes in *sgk1* in these brain regions following fear-conditioning although previous studies have demonstrated, that *sgk1* was expressed and functionally important in the hippocampus during fear consolidation (von Hertzen *et al.*, 2005) (Lee *et al.*, 2007). It is possible that changes in *sgk1* expression in these brain regions may occur at a different time point to that examined in the present study or following a different form of stress.

The present experiments assessed whether changes (behavioural such as freezing or FCA, or molecular) during re-exposure to the conditioned stressor are associated with conditioned fear *per se* or with footshock exposure the previous day. Although a number of important contextual variables (such as light, sound, smell, colour) were manipulated to distinguish the fear conditioning chambers, rats fear-conditioned in this different context (i.e. FCD rats) still expressed some freezing behaviour and 22kHz ultrasonic vocalisation when re-exposed to the ‘conventional’ fear conditioning chamber, though this was much lower in magnitude and short-lived. This fear behaviour may be due to some similarities between the arenas, for example similarities in shape. In addition, the similar process of carrying rats from their cages 24hrs after they received footshock and placing them in another novel arena by itself could retrieve the fear memory albeit to magnitude lesser extent than re-exposing them to the arena with the appropriate cues. Together with this brief expression of freezing and 22KHz ultrasonic vocalisation, FCD rats showed minimal FCA that was limited to the first 5min of the trial. Despite the expression of FCA, there were no associated alterations in expression of either *zif268* or *sgk1* in any of the regions investigated. Therefore, molecular changes that accompany FCA appear to be due to the conditioned fear *per se* rather than the footshock exposure the previous day. Although conditioned fear was associated with a suppression of total activity (sum duration of rearing, grooming and walking), the reduction in pain-related behaviour following fear conditioning does not appear to be due to reduced activity because AM251 did not affect total activity, despite attenuating FCA.

In conclusion, this study demonstrated that fear-induced suppression of formalin-evoked nociceptive behaviour was associated with attenuation of formalin-evoked increased expression of *zif268* in the ipsilateral DHSC. Pharmacological blockade of the CB<sub>1</sub> receptor prevented FCA, and attenuated fear-induced suppression of *zif268* expression in the DHSC. These data provide strong evidence for *zif268* expression in the DHSC as a molecular correlate of endocannabinoid-mediated FCA. Furthermore, conditioned-fear was accompanied by increased expression of *zif268* in the RVM and a strong similar trend in the amygdala, supporting and extending previous evidence for a role of the RVM and amygdala in conditioned fear behaviour and suggests *zif268* as an important molecular correlate.

***Chapter 5: Characterisation of the endocannabinoid system in discrete brain regions in a rat model of anxiety-related hyperalgesia***

### **5.1 Introduction**

The ability of stress, anxiety and fear to suppress pain responding and induce stress-induced or fear-conditioned analgesia (SIA/FCA) has long been recognized as an adaptive/defence response allowing the organism to cope with immediate threats and has been the focus of the work presented in this thesis so far. However, stress/anxiety does not invariably suppress pain; depending on their nature, duration and intensity, stress or anxiety may also exacerbate pain, a phenomenon referred to herein as SIH or ARH. There has been growing interest in the interaction between persistent pain and stress/anxiety. Clinically, stress has a major impact on pain perception (Bennett *et al.*, 1998; Conrad *et al.*, 2007; Fishbain *et al.*, 2006; Grande *et al.*, 2004; Nilsen *et al.*, 2007; Zautra *et al.*, 2007). Psychiatric conditions, including anxiety disorders, are associated with an increased frequency of chronic pain complaints (Asmundson *et al.*, 2009; Atkinson *et al.*, 1991; Dworkin *et al.*, 1995; Kain *et al.*, 2000; Lautenbacher *et al.*, 1999), and experimentally induced anxiety is also known to enhance pain sensitivity in humans (Al Absi *et al.*, 1991; Cornwall *et al.*, 1988; Dougher, 1979; Rhudy *et al.*, 2000; Schumacher *et al.*, 1984). Stressors such as novelty, vibration, social threat, water avoidance stress, restraint stress, forced swim and exposure to cold have all been shown to induce hyperalgesia in animal models of pain (Bradesi *et al.*, 2005; da Silva Torres *et al.*, 2003b; Hayes *et al.*, 1978; Langford *et al.*, 2011; Quintero *et al.*, 2000; Satoh *et al.*, 1992; Vidal *et al.*, 1982). While there is convincing evidence for SIH/ARH and the clinical burden it imposes, a paucity of data exists regarding the neurochemical and molecular mechanisms mediating this phenomenon. Development of animal models of SIH/ARH is central to increasing our understanding of the pathophysiological mechanisms underlying this association and to the potential discovery of effective new therapeutic targets.

WKY rats are a genetic variation of the Wistar strain (Okamoto and Aoli, 1963) which demonstrate hormonal, behavioural, and physiological changes that resemble alterations seen during stress/anxious state and depression. For

instance, WKY rats exhibit anxiogenic-like profile during exposure to aversive environments (Gentsch *et al.*, 1987; Paré, 1994). Moreover, WKY rats are hyper responsive to stress (De La Garza II *et al.*, 2004; Lahmame *et al.*, 1997; Malkesman *et al.*, 2005; Rittenhouse *et al.*, 2002; Tejani-Butt *et al.*, 1994) and exhibit neuroendocrine differences including an enhanced HPA axis response to stress (Rittenhouse *et al.*, 2002; Solberg *et al.*, 2001) when compared to other strains commonly used as a control comparator, such as SD rats. WKY rats also have depressive-related phenotype exhibited by increased immobility in the forced swim test (Porsolt, 1977). For these reasons, WKY rats have been proposed as a model of stress vulnerability and high trait anxiety.

In addition to the anxiogenic phenotype, increased sensitivity of WKY rats to both visceral and somatic noxious stimuli has been reported. For example, WKY rats are hyper responsive to visceral stimuli such as colorectal distention (Gibney *et al.*, 2010; Greenwood-Van Meerveld *et al.*, 2005; Gunter *et al.*, 2000; O'Mahony *et al.*, 2010) or urinary bladder distention (Robbins *et al.*, 2007). Compared to Wistar rats, WKY rats exhibit enhanced mechanical allodynia following peripheral nerve injury (Zeng *et al.*, 2008). WKY rats also exhibit a hyperalgesic response in the paw withdrawal test (Taylor *et al.*, 2001), intra-plantar formalin-induced inflammatory pain model (Burke *et al.*, 2010) and hot plate test (Burke *et al.*, 2010) when compared with SD rats. However, conflicting results have also been reported (Taylor *et al.*, 2001). These authors reported no difference in nociceptive responding between SD and WKY rats in the hot plate, tail flick and formalin tests (Taylor *et al.*, 2001). Burke and colleagues also reported no difference in nociceptive responding between SD and WKY rats in the tail flick test (Burk *et al.*, 2010). Moreover, intra-plantar injection of zymosan reduced thermal latency and mechanical threshold to a greater extent in SD rats compared to WKY rats (Taylor *et al.*, 2001). Nevertheless, the weight of evidence indicates strongly that WKY rats display an increased sensitivity to noxious stimuli and this phenotype, coupled with their anxiogenic profile, suggests that they could constitute a useful genetic model of ARH.

The endocannabinoid system is a recently identified neuromodulatory system, which is involved in several physiological processes including pain and anxiety. CB<sub>1</sub> receptors in a number of corticolimbic and brainstem structures are implicated in the regulation of fear- and pain-related behaviours (Hoot *et al.*, 2010; Lin *et al.*, 2009; Martin *et al.*, 1999; Bradshaw *et al.*, 2006; Campos *et al.*, 2010; de Oliveira Alvares *et al.*, 2010; Hasanein *et al.*, 2007; Manning *et al.*, 2003; Marsicano *et al.*, 2002; Martin *et al.*, 1999; Roche *et al.*, 2007; Finn *et al.*, 2003; Lisboa *et al.*, 2008; Moreira *et al.*, 2009a; Ressell *et al.*, 2008; Walker *et al.*, 1999; Wilson *et al.*, 2008). As such, in addition to playing a role in stress/fear-induced analgesia, the endocannabinoid system may also play an important role in SIH/ARH. Indeed, Shen and colleagues showed that intraperitoneal administration of CB<sub>1</sub> receptor agonist, ACEA, significantly diminished partial restraint stress-induced visceral hypersensitivity to colorectal distension, whereas, the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, further enhanced the hyperalgesia (Shen *et al.*, 2010). Treatment of water avoidance-stressed rats with the CB<sub>1</sub> receptor agonist, WIN 55,212-2, prevented the development of visceral hyperalgesia, suggesting a potentially important role for the endocannabinoid system in stress-induced visceral hyperalgesia (Hong *et al.*, 2009). Alterations in endocannabinoid levels have been reported following exposure of rats or mice to acute or chronic aversive or painful stimuli in various brain regions including the PAG (Hohmann *et al.*, 2005; Petrosino *et al.*, 2007; Walker *et al.*, 1999), amygdala (Marsicano *et al.*, 2002; Patel *et al.*, 2005; Rademacher *et al.*, 2008), hypothalamus (Patel *et al.*, 2004), PFC (Gorzalka *et al.*, 2008; Rademacher *et al.*, 2008), hippocampus (Hill *et al.*, 2005), dorsal raphe, spinal cord and RVM (Petrosino *et al.*, 2007). However, the response of the endocannabinoids and related lipids in relevant brain regions during ARH has not been investigated.

*In situ* hybridization and immunostaining studies have demonstrated heterogeneous distribution of CB<sub>1</sub> receptors throughout the CNS including the cerebral cortex, basal ganglia, cerebellum, amygdala, PAG, pons and medulla (Freund *et al.*, 2003; Herkenham *et al.*, 1991) and spinal cord (Ahluwalia *et al.*, 2002; Farquhar-Smith *et al.*, 2000; Salio *et al.*, 2002). Similarly, the expression of the catabolic enzyme, FAAH which metabolises AEA, PEA and OEA

(Cravatt *et al.*, 1996), is localised in the cerebral cortex, hippocampus, cerebellum, olfactory bulb, pituitary gland, striatum and hypothalamus (Thomas *et al.*, 1997). Moderate immuno-staining has also been observed in the amygdala, basal ganglia, ventral and posterior thalamus, the brain stem and the spinal cord (Freund *et al.*, 2003; Thomas *et al.*, 1997; Tsou *et al.*, 1998). Many of these FAAH-positive neurons in the brain are found in proximity to nerve terminals that contain CB<sub>1</sub> receptors (Thomas *et al.*, 1997). Similarly, moderate to high levels of *MAGL mRNA* are expressed throughout the rat brain including the cerebral cortex, thalamus, cerebellum, striatum, hippocampus, hypothalamus and the brain stem (Dinh *et al.*, 2002) and the spinal cord (Garcia-Ovejero *et al.*, 2009). As is the case with *FAAH mRNA*, *MAGL mRNA* and protein mirrors that of CB<sub>1</sub> receptors in the CNS (Dinh *et al.*, 2002).

Chronic stress has been shown to result in alterations in the expression of CB<sub>1</sub>, FAAH and MAGL. For example, chronic stress downregulated CB<sub>1</sub> receptor expression in the hippocampus (Hill *et al.*, 2005; Reich *et al.*, 2009) without affecting levels in the limbic forebrain (Hill *et al.*, 2005) in male rats. Conversely, this chronic stress procedure induced upregulation of CB<sub>1</sub> receptors in the hippocampus of female rats (Reich *et al.*, 2009). In an alternative stress model, CB<sub>1</sub> receptor mRNA expression levels were shown to be higher in the prefrontal cortex, other cortical layers and thalamus of socially isolated rats (Robinson *et al.*, 2010). Chronic stress also produced an upregulation of FAAH levels in the hippocampus (Reich *et al.*, 2009). In addition, FAAH mRNA expression was shown to be lower in prefrontal cortex and the caudate putamen; whereas, MAGL mRNA expression levels were higher in the prefrontal cortex and thalamus of socially isolated rats (Robinson *et al.*, 2010). Maternal deprivation induced a decrease in MAGL immunoreactivity and MAGL mRNA levels in the hippocampus (Suárez *et al.*, 2010). However, the extent to which the levels of expression of *CB<sub>1</sub>*, *FAAH*, and *MAGL mRNA* in discrete regions of CNS vary between SD and WKY rats, and possibly contribute to their differential response to stress and pain, is unknown.

The aims of the experiments described in this chapter are:

- to characterise a model of trait anxiety-related hyperalgesia using WKY versus SD rats, two strains with different in baseline emotionality
- to determine levels of endocannabinoids and related lipids (NAEs) in discrete brain regions of SD and WKY rats, in the presence and absence of nociceptive tone
- to measure the levels of expression of CB<sub>1</sub> receptor, *FAAH* and *MAGL mRNA* in discrete brain regions and in the dorsal horn of the spinal cord in SD and WKY rats

The work tests the hypothesis that WKY rats are more anxious and hyperalgesic to thermal and inflammatory noxious stimuli compared with SD rats and that these behavioural differences are due to differences in the expression and activity of the endocannabinoid system in the two strains.

## **5.2 Methods**

### **5.2.1 Animals**

Experiments were carried out in adult male SD (n=24) and WKY (n=24)(285-320g) rats (Harlan, UK), all singly housed and maintained at a constant temperature ( $21 \pm 2^{\circ}\text{C}$ ) under standard lighting conditions (12:12h light:dark, lights on from 07.00 to 19.00h). All experiments were carried out during the light phase between 08.00h and 17.00h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

### **5.2.2 Experimental design**

The experimental procedure consisted of four tests. On test day 1 and 2 rats were tested in the open field and elevated plus maze, respectively, to examine anxiety-related behaviours. On test day 3, rats were tested on the hot plate test to assess their response to a noxious thermal stimulus. Subjects were assigned to two groups, SD and WKY (n=24 per group), and the sequence of group testing was randomized in order to minimize any confounding effects of testing procedure. On day 6-9 rats were assessed in the formalin test where subjects received an intra-plantar injection of 50 $\mu\text{L}$  formalin (2.5% in 0.9% saline) or 0.9% saline (control group) into the right hind-paw. This design resulted in four experimental groups: SD-Saline (SD-Sal), SD-Formalin (SD-Form), WKY-Saline (WKY-Sal) and WKY-Formalin (WKY-Form) (n=12 per group). At the end of the formalin test (i.e. 30min post-formalin injection), rats were killed by decapitation. Brains and spinal cords (L4-L6 lumbar enlargement ipsilateral and contralateral dorsal quadrants) were removed rapidly, snap-frozen on dry ice and stored at -80 $^{\circ}\text{C}$  prior to endocannabinoid/NAE assay by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) or mRNA analysis by RT-qPCR.

### **5.2.3 Behavioural testing**

#### **5.2.3.1 Open field test**

Behaviour in the open field was assessed once for SD and WKY rats in an alternate manner on the day 7 post arrival. On the experiment day, each animal was removed from the home cage during the light phase between 0900 h and 1400 h and placed into a brightly lit (lux 300) novel white open field environment (diameter 75 cm and 40cm high walls). A camera positioned 35cm above the floor allowed for behaviour to be captured, recorded and assessed using a computerized video tracking system (EthoVision® XT7, Noldus, The Netherlands) for a 5 min period. The open field was cleaned between animals with cleaning solution (Milton:tap water; 1:5). Behaviours assessed included locomotor activity (total distance moved and distance moved in the centre; cm) and duration of time spent (duration; s) in the centre zone (45 cm diameter). Time spent in the centre zone is usually interpreted as anxiety-related behaviour.

#### **5.2.3.2 Elevated plus maze (EPM)**

The elevated plus maze consisted of a plus-shaped wooden maze with two closed arms enclosed by walls (30 cm) and two open arms. Each arm was 50 cm in length and 10 cm in width, and the arms were inter-connected by a central platform and elevated 50 cm from the room floor. A video camera was positioned over the maze and the light levels were fixed at 60 lux in the open arms and 25 lux in the closed arms. The EPM test was carried out once for both WKY and SD in a randomised fashion. On the experiment day (day 8 post arrival), rats were placed on the central platform with their head pointing towards one of the open arms. The rat behaviour was recorded and analyzed using a computerized video tracking system (EthoVision® XT7, Noldus, the Netherlands) for a 5 min period. The EPM was cleaned between animals with cleaning solution (Milton: tap water; 1:5). Time spent in the open arms(s) and percentage of entries in the open arms in relation to the total number of arm entries were used as experimental indices of anxiety, whereas the entries in the closed arms are seen as indices of general locomotion. Entries in arms were defined as entry of the rat's centre of gravity into the arms. Distance moved in each section of the EPM and the percentage distance moved in the OA of EPM was also assessed.

### **5.2.3.3 Nociceptive responding**

#### **5.2.3.3.1 Hot plate test**

Nociceptive responding of SD and WKY rats to an acute thermal stimulus was assessed using the hot plate test. Nociceptive responding in the hot plate test is predominantly supraspinally mediated, requiring activation of the medial prefrontal cortex in order to elicit a response (Pastoriza *et al.*, 1996). Hot plate testing was carried out once for SD and WKY rats on day 9 post arrival. The animal was taken from its home cage and placed directly onto a hot plate (IITC Life Science Inc, CA, USA) heated to  $55\pm2$  °C. Thermal nociception was measured as the time elapsed (i.e. latency to respond (s) between placements of the animal on the surface of the hot plate and when the animal first licked and/or withdraw either of its hind paws, with a cut-off time of 40s to avoid tissue damage. The hot plate was cleaned between animals with cleaning solution (Milton:tap water; 1:5).

#### **5.2.3.3.2 Formalin-induced nociceptive behaviour**

Nociceptive behaviour in the formalin test was assessed for each animal on day 12-15 post arrival. Rats were placed in a Perspex observation chamber (30x30x40 cm<sup>3</sup>) (lux of 30) for a 10 min habituation after which time they received an intra-plantar injection of 50 µL formalin (2.5% in 0.9% saline) or 0.9% saline into the right hind paw under brief isoflurane anaesthesia as previously described (Finn *et al.*, 2003; Roche *et al.*, 2007b, 2010; Chapters 2, 3 & 4). Rats were returned to their home cage for a further 3 min, at which point they were returned to the same Perspex observation chamber to which they had been previously exposed. Behaviour was recorded for 30 min from a video camera located beneath the observation chamber. The chamber was cleaned between animals with cleaning solution (Milton:tap water; 1:5). Behaviour was analysed with the aid of EthoVision®XT7 software by a rater blind to experimental conditions. Formalin-evoked nociceptive behaviour was scored according to the weighted composite pain scoring technique (Watson *et al.*, 1997). According to this method, pain behaviours are categorized as time spent raising the formalin injected paw above the floor without contact with any other surface (C1) and holding, licking, biting, shaking or flinching the injected paw

(C2) to obtain a composite pain score ( $CPS = (C1 + 2(C2))/(total\ duration\ of\ analysis\ period)$ ). Formalin-induced oedema was assessed by measuring the change in the diameter of the right hind paw immediately before and 30min after formalin administration using Vernier callipers. Locomotor activity (duration of rearing and grooming) and duration of freezing (defined as the duration spent without movement except movement required for respiration) during the pre-trial 10 min period were also rated manually, and total distance moved was determined automatically at the same time using Ethovision XT7 tracking. Rats were killed by decapitation 30min post-formalin injection and brains and spinal cords were removed and snap-frozen on dry ice as described in section 5.2.2. Brains were stored at -80°C prior to punch dissection of cryosectioned brain regions for endocannabinoid/NAE assay by mass spectrometry or mRNA analysis by RT-qPCR.

#### **5.2.4 Cryo-sectioning**

Frozen coronal brain sections (300µm thickness) containing the PAG, BLA, hippocampus, medial prefrontal cortex (mPFC), insular cortex and RVM were cut on a cryostat (MICROM GMBH, Germany). The following tissues were punched from the frozen sections using cylindrical brain punches (Harvard Apparatus, internal diameter 0.75-2mm; 0.75mm for the different PAG regions and BLA, and 2mm for the other regions): mPFC (Bregma, 3.7 — -0.3mm), insular cortex (Bregma, 2.7 — -0.7mm), dorsalhippocampus (dHipp; Bregma, -1.8 — -8.0mm), ventralhippocampus (vHipp; Bregma, -7.3 — -8.0mm), BLA (Bregma, -1.8 — -3.3mm), dlPAG (Bregma, -5.8 — -8mm), vlPAG (Bregma, -7.3 — -8.3mm), lateral(l)PAG (Bregma, -7.3 — -8.3mm), and RVM (Bregma, -9.16 — -11.6mm). In order to investigate the effect of lateralisation, separate punches were taken for left and right for all regions mentioned above except RVM and mPFC. Punched brain regions were weighed (range of weights of punched tissue: 4.5-20 mg depending on the region) and stored at -80°C prior to extraction for determination of the concentrations of the endocannabinoids or NAEs by LC-MS/MS or mRNA extraction for RT-qPCR.

### **5.2.5 Quantitation of endocannabinoids and NAEs in discrete brain regions using LC-MS/MS**

Levels of endocannabinoids and NAEs in the dlPAG, vlPAG, IPAG, BLA, vHipp ,dHipp, mPFC, insular cortex and RVM were analysed using the LC-MS/MS method described in Section 2.2.5. Both left and right side of these regions (except RVM and mPFC) were analysed for the four experimental groups (i.e. SD-Sal, SD-Form, WKY-Sal and WKY-Form, n=6 per group)

### **5.2.6 Real Time-Quantitative Polymerase chain reaction (RT-qPCR)**

#### **5.2.6.1 RNA preparation**

Brain tissues from dlPAG, vlPAG, IPAG, BLA, RVM and DHSC of the two saline-treated experimental groups (i.e SD-Sal and WKY-Sal, n=6) were analysed by RT-qPCR. Total RNA was extracted from homogenized tissue using a Machery-Nagel extraction kit (Nucleospin RNA II, Technopath, Tipperary, Ireland). This method involved homogenising tissue in 350µl lysis buffer (RA1), containing 1% β-mercaptoethanol (Sigma, Dublin, Ireland ) for 3s using an automated homogeniser (Polytron tissue disrupter, Ultra-Turrax, Germany). Homogenates were kept on ice until transferred to a Nucleospin filter (violet ring), centrifuged at 11000g for 1min and the lysates treated with 350µl of 70% molecular grade ethanol (Sigma, Dublin, Ireland). Samples were transferred to Nucleospin RNA spin column II (light blue ring) and centrifuged at 11000g for 30s to bind the RNA. After desalting the column membrane with membrane desalting buffer (MDB,), RNA samples were treated with 10µl DNase for 15min at room temprature to remove DNA from the sample. Samples were then serially washed using washing buffers (200µl RA2, 600µl RA3 and 250µl RA3;) and RNA was eluted in 20µl of RNAase-free water (Sigma, Dublin, Ireland). The quantity, purity and quality of RNA were assessed using Nanodrop (ND-1000, Nanodrop, Labtech International, Ringmer, UK). RNA quantity was determined by measuring optical density (OD) at 260nm. RNA quality was determined by measuring the ratio OD<sub>260</sub>/OD<sub>280</sub> where a ratio of approximately 1.8-2.1 was deemed indicative of pure RNA. All mRNA samples showed OD<sub>260</sub>/OD<sub>280</sub> ratios between 1.75 and 2.2 on the Nanodrop. mRNA samples were kept at -80<sup>0</sup>C until required for cDNA synthesis.

### **5.2.6.2 cDNA synthesis**

cDNA synthesis was carried out as described in section 4.2.5.2 of Chapter 4.

### **5.2.6.3 cDNA amplification**

The cDNA was used as the template for real-time quantitative PCR (RT-PCR), which was performed on the AB7500 PCR system (Applied Biosystems 7500) using TaqMan gene expression assays containing specific target primers for *GAPDH*, *CB1*, *FAAH* and *MAGL* and 6-carboxyfluorescein (FAM)- or VIC-labelled probes (Applied Biosystems, Dublin, Ireland) (see Table 5.1 for primers sequences). *GAPDH* gene expression was used to normalize gene expression between samples and was quantified using a *GAPDH* endogenous control assay containing specific primers and a VIC-labelled probe. Previously prepared cDNA samples were diluted 1:4 and 10 $\mu$ l of each diluted sample was pipetted onto a MicroAmp<sup>TM</sup> optical 96 well plate (Applied Biosystems, Dublin, Ireland). cDNA was amplified in a reaction that contained 10 $\mu$ l of cDNA template, 1.25 $\mu$ l of the primers for the target gene and 1.25 $\mu$ l of the primers for the endogenous control gene (i.e. *GAPDH*), and 12.5 $\mu$ l of TaqMan Universal Master Mix in a final reaction volume of 25 $\mu$ l in a three-step cycling program. Control cDNA samples (obtained without transcriptase or RNA) were always included, as well as samples without any cDNA template. Plates were covered with adhesive plate covers and spun at 1000g for 10s to ensure complete mixing. Cycling parameters were 50<sup>0</sup>C for 2min to activate DNA polymerase, 95<sup>0</sup>C for 1min to denature DNA. This was followed by 40 cycles of 95<sup>0</sup>C for 15s and 60<sup>0</sup>C for 1min, in which fluorescence was acquired.

Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems 7500 SDS Software 1.3.1. Reactions were performed for each sample and Ct values were normalized to the housekeeping *GAPDH* gene expression. The relative expression of target genes to *GAPDH* was calculated by using the  $2^{\Delta Ct}$  method. In this method,  $\Delta Ct = Ct$  for the reference gene in the test sample – Ct for the target gene in the test sample. The  $2^{\Delta Ct}$  values for each sample were then expressed as a percentage of the mean of the  $2^{\Delta Ct}$  values for the control group (NFC-Sal). In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

**Table 5.1 Sequence of primers for RT-qPCR**

<b>Gene</b>	<b>Probe</b>	<b>Assay ID</b>	<b>Melting temperature (°C)</b>
GAPDH	VIC	4308313	63.4
CB <sub>1</sub>	FAM	Rn00562880_m1	62
FAAH	FAM	Rn00577086_m1	46.5
MAGL	FAM	Rn00593297_m1	58.1

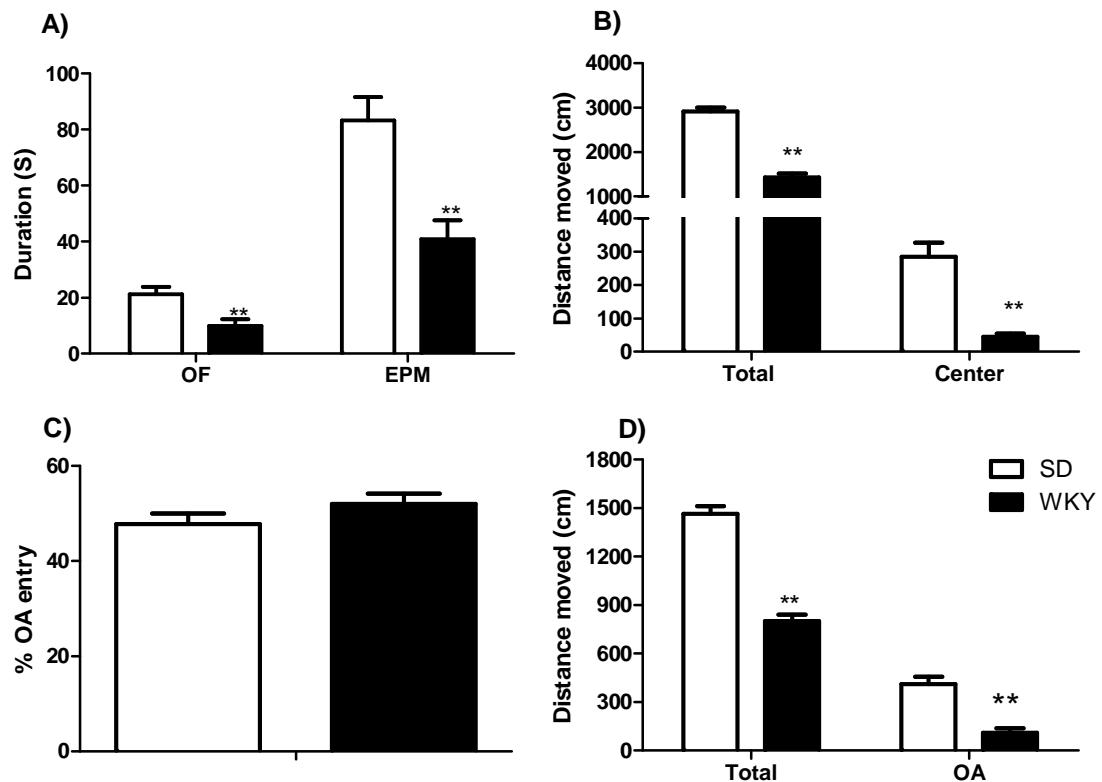
### **5.2.7 Statistical analysis**

The SPSS 17.0 statistical package was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene test, respectively. Student's unpaired, two-tailed T-test was used to compare the hot plate, open field, EPM and gene expression data between SD and WKY rats (section 5.3). Formalin test data and neurochemical data were analysed using two-factor analysis of variance (ANOVA), with the factors of strain and formalin. Timecourse behavioural data were analysed by repeated measures ANOVA with time as the within-subjects factor and group as the between-subjects factor. Post-hoc pairwise comparisons were made with Fisher's LSD when appropriate. Data are expressed as group means ± standard error of the mean (± SEM) and were considered significant when P<0.05.

### **5.3 Results**

#### **5.3.1 Comparison of anxiety-related behaviour in WKY and SD rats**

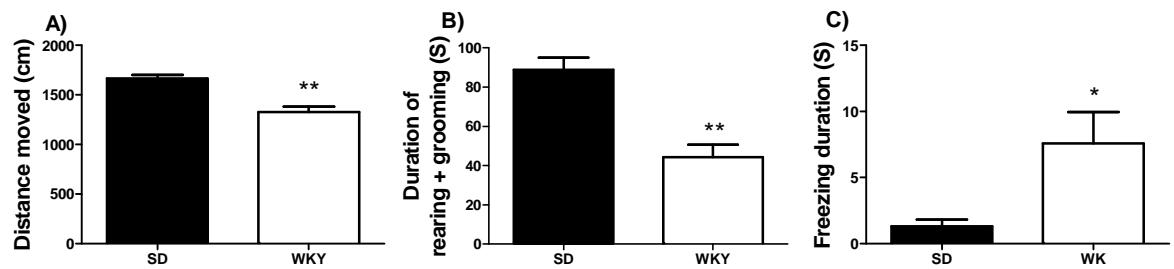
In the open-field test, WKY rats spent significantly less time in the centre zone of the open-field (**Fig. 5.1A**) and exhibited significantly decreased locomotor activity (i.e. decrease in total distance moved and distance moved in the centre zone; **Fig 5.1B**) when compared with SD counterparts (SD vs. WKY,  $p<0.01$ ). In the EPM test, data analysis revealed no statistically significant difference in the percentage of open arm entries (% OA Entry) between the two strains (**Fig. 5.1C**). However, WKY rats spent significantly less time on the open arms of the elevated plus maze (SD vs. WKY,  $p<0.01$ , **Fig 5.1A**) and demonstrated hypoactivity as shown by both total distance and distance moved in the open arms compared to SD rats (Total distance, distance moved OA; SD vs. WKY,  $p<0.05$ , **Fig 5.1D**). There was no difference between the two strains in the number of entries into the closed arm, an index of general locomotion (% closed arm entry; SD, 50.20 vs. WKY, 48.81).



**Fig 5.1** Open-field (OF) and elevated plus maze (EPM) behaviours in SD and WKY rats, (A) time spent in the center zone of the OF:  $t_{46}=3.19$ ,  $p<0.01$  and in the open arms of the EPM:  $t_{46}=4.06$ ,  $p<0.01$  (B) distance moved in the OF (Total:  $t_{46}=11.75$ ,  $p<0.01$ , Centre:  $t_{46}=5.67$ ,  $p<0.01$ ); (C) % OA entries on the EPM:  $t_{46}=1.68$ ,  $p=0.10$ , (D) distance moved on the EPM (total:  $t_{46}=7.6$ ,  $p<0.01$ , OA:  $t_{46}=7.43$ ,  $p<0.01$ ); unpaired t-test \* $p<0.05$ , \*\* $p<0.01$  vs. SD; Data are mean  $\pm$  SEM ( $n=24$ ); SD, Sprague Dawley; WKY, Wistar Kyoto; OA, open arms; EPM, elevated plus maze

### 5.3.3 Comparison of general locomotor activity and freezing behaviours in a novel environment between WKY and SD rats pre-formalin injection

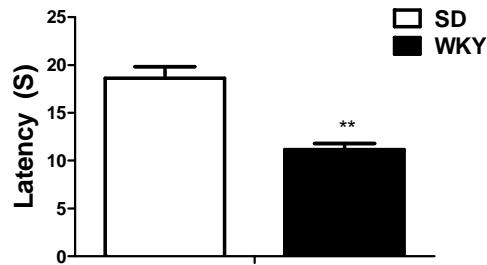
During the 10min habituation period in the novel Perspex arena prior to intra-plantar formalin/saline administration, WKY rats displayed significantly lower locomotor activity as measured by the distance moved using Ethovision tracking system or the duration of manually scored behaviours (i.e. sum of duration of rearing and grooming) assessed manually when compared to SD rats (SD vs. WKY,  $p<0.01$ , **Fig 5.2A&B**). During this time, although both WKY and SD rats showed minimal freezing behaviour, WKY rats showed increased duration of freezing when compared with SD rats (SD vs. WKY,  $p<0.05$ , **Fig 5.2C**).



**Fig 5.2** Exploratory/locomotor and freezing behaviours of SD and WKY rats during the 10min pre-formalin injection trial period; A) distance moved using automated Ethovision tracking  $t_{40}=5.2$ ,  $p<0.01$ ; B) sum duration of rearing and grooming:  $t_{43}=4.99$ ,  $p<0.01$ ; unpaired t-test \*\*  $P<0.01$  vs. SD; (C) duration of freezing:  $t_{43}=2.52$ ,  $p<0.05$ ; unpaired t-test \*\* $p<0.01$  vs. SD; Data expressed as mean  $\pm$  SEM ( $n=24$ ).

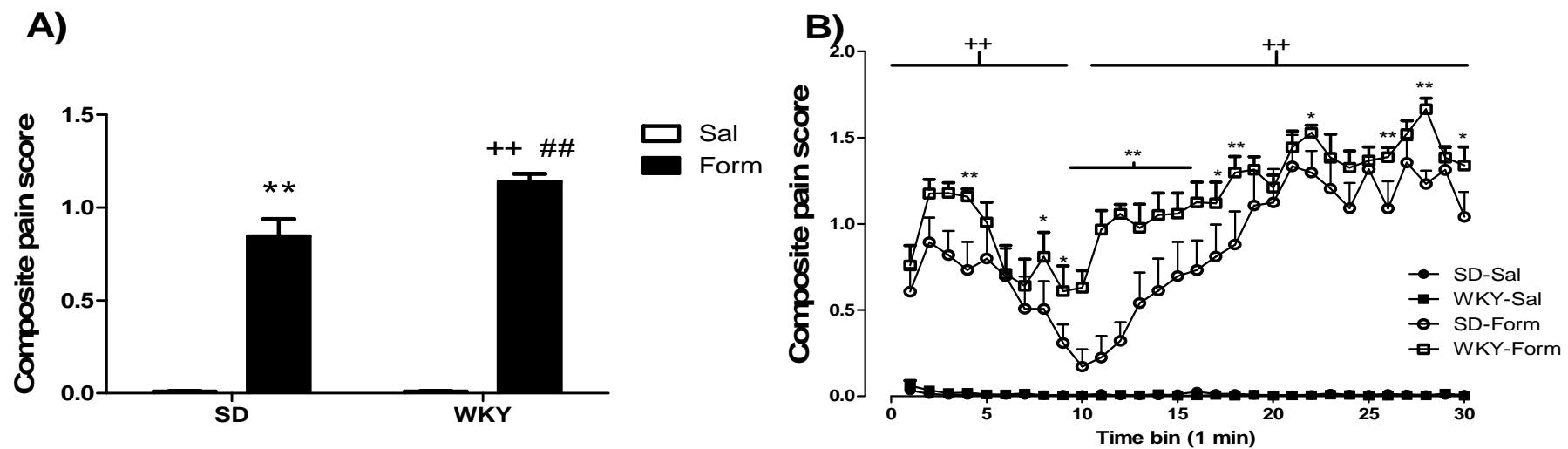
### 5.3.2 Nociceptive responding of WKY and SD rats to noxious thermal and persistent inflammatory stimuli

WKY rats showed reduced latency to lick/withdraw either of their hind paws on the hot plate test, compared with the SD rats ( $p<0.01$ , **Fig 5.3**).



**Figure 5.3** Nociceptive responding to acute thermal stimulation on the hot plate test ( $55\pm2^{\circ}\text{C}$ ; unpaired t-test  $t_{46}=5.45$ , \*\*  $p<0.01$  vs. SD; Data expressed as mean $\pm$ SEM ( $n=24$ ); SD, Sprague-Dawley; WKY, Wistar-Kyoto

Compared with controls receiving intra-plantar injection of saline, intra-plantar formalin administration produced robust licking, biting, shaking and elevation of the right hind paw as indicated by the composite pain score (**Fig. 5.4A and B**). Both SD and WKY rats exhibited the classic biphasic formalin response (**Fig. 5.4B**) demonstrated by a peak in nociceptive behaviour approximately 1-3min into the trial, which subsides and is then followed by a second phase of nociceptive behaviour approximately 11-13 min into the trial. Analysis revealed that WKY rats exhibited significantly higher formalin-evoked nociceptive behaviour over the 30min trial period (SD-Form vs. WKY-Form,  $p<0.01$ , **Fig 5.4A**). This effect was seen throughout the 30min trial including the first phase, inter-phase and second phase of formalin-induced nociception (SD-Form vs. WKY-Form,  $p<0.05$ , **Fig 5.4B**). During the inter-phase period (7-14min), CPS of formalin-injected SD rats almost lowered to a level close to that of saline-injected rats but CPS of formalin-injected WKY rats remained higher. In addition, assessment of area under the curve revealed that WKY rats exhibit enhanced nociceptive responding following intra-plantar formalin injection when compared to SD controls over the 30min period (SD-Form,  $24.55\pm1.36$ min vs. WKY-Form,  $33.17\pm1.67$ min ,  $p<0.05$ ). Thus, WKY rats display a significant increase in the magnitude of formalin-evoked nociceptive behaviour throughout the formalin test trial.



**Figure 5.4** Effects of intra-plantar injection of saline or formalin on nociceptive behaviour in SD and WKY rats A) Total over 30 min: (ANOVA: strain:  $F_{(1, 41)}=9.9$ ,  $p<0.001$ ; formalin:  $F_{(1, 41)}=441.3$ ,  $p<0.001$  and formalin x strain interaction:  $F_{(1, 41)}=9.94$ ,  $p=0.003$ ), \*\* $p<0.01$  vs. SD-Sal, ++ $p<0.01$  vs. SD-Form & ## $p<0.05$  vs. WKY-Sal, B) 1 min time bin: (repeated measures ANOVA time:  $F_{(29, 1189)}=15.74$ ,  $p<0.001$ ; time x formalin:  $F_{(29, 1189)}=16.00$ ,  $p<0.001$ , and time x strain interaction:  $F_{(29, 1189)}=1.27$ ,  $p=0.15$ ; time x strain x formalin:  $F_{(29, 1189)}=1.31$ ,  $p=0.13$ ); \* $p<0.05$ , \*\* $p<0.01$  SD-Form vs. WKY-Form, ++ $p<0.01$  SD/WKY-Sal vs. SD/WKY-Form; Data are mean  $\pm$  SEM ( $n=12$ ); CPS, composite pain score; SD, Sprague-Dawley; WKY, Wistar-Koyoto; Sal, Saline; Form, Formalin.

### **5.3.3 Comparison of general locomotor activity, defecation and change in paw diameter between WKY and SD rats post-formalin injection**

Intra-plantar injection of formalin induced right-hind paw oedema in both strains when compared with saline-injected controls (SD/WKY-Form vs. SD/WKY-Sal, **Table 5.2**). There was no significant difference in change in paw diameter (i.e. post-formalin minus pre-formalin paw diameter) between SD and WKY rats. WKY rats (saline- or formalin-treated) excreted significantly more faecal pellets during the formalin test trial compared to respective SD controls (SD-Sal/Form vs. WKY-Sal/Form,  $p<0.01$ , **Table 5.2**). WKY rats receiving intra-plantar saline injection showed a decrease in the sum duration of rearing + grooming compared to SD counterparts though this result did not reach statistical significance (SD-Sal vs. WKY-Sal, **Table 5.2**). Formalin injection significantly reduced the sum duration of rearing + grooming in both SD and WKY rats (SD/WKY -Form vs. SD/WKY -Sal,  $p<0.01$ , **Table 5.2**). However, no difference in the sum duration of rearing + grooming was seen between formalin-injected SD and WKY rats (SD-Form vs. WKY-Form, **Table 5.2**). Locomotor activity measured using automated Ethovision tracking system showed an increase in total distance moved following intra-plantar formalin injection in both strains compared to their respective saline treated controls (SD/WKY-Form vs. SD/WKY -Sal,  $p<0.05$ , **Table 5.2**). In addition, in the same measurement, both saline- and formalin-injected WKY rats showed significantly lower locomotor activity than their SD counterparts (WKY-Sal/ Form vs. SD-Sal/ Form,  $p<0.05$ , **Table 5.2**). WKY and SD rats receiving either intra-plantar saline or formalin did not differ in the duration of freezing behaviour over the 30min trial period (SD-Sal/Form vs. WKY-Sal/Form, **Table 5.2**). Formalin injection also did not have a statistically significant effect on the duration of freezing in SD rats when compared with saline-injected controls (SD-Sal vs. SD-Form, **Fig 5.3F**). However, WKY rats that received formalin displayed a significantly lower duration of freezing behaviour when compared to saline-treated counterparts (WKY-Sal vs. WKY-Form,  $p<0.05$ , **Table 5.2**).

Groups	Δ Paw diameter (mm)	Defecation (number of pellets)	The sum duration of rearing + grooming (S)	Distance moved (cm)	Freezing (S) Mean±SEM
	Mean±SEM	Mean±SEM	Mean±SEM		
<b>SD-Sal</b>	0.68±0.07	2.42±0.34	81.65±12.47	1417.58±106.81	168.18±74.15
<b>WKY-Sal</b>	0.56±0.04	6.58±0.54**	62.28±13.25	955.77±97.88**	228.15±66.45
<b>SD-Form</b>	1.52±0.06**	2.75±0.28	30.06±9.44**	2454.33±325.38**	110.16±30.57
<b>WKY-Form</b>	1.4±0.07**	5.83±0.58##	22.48±5.25**	1475.89±106.28*##	40.10±15.72*

**Table 5.2** Effect of formalin on hind paw oedema, defecation, general exploratory/locomotor and freezing behaviours in SD and WKY rats during the 30 min post-formalin trial period (ANOVA: Hind paw oedema: strain;  $F_{(1,44)}=3.77$  p=0.057, formalin;  $F_{(1,44)}=81$ , p<0.01, strain x formalin  $F_{(1,44)}=0.00$ , p=0.95; Defecation: strain;  $F_{(1,44)}=64.39$ , p<0.01, formalin;  $F_{(1,44)}=0.23$ , p= 0.642, strain x formalin  $F_{(1,44)}=1.44$ , p=0.24; Sum duration of rearing + grooming: strain  $F_{(1,41)}=1.6$ , p=0.213, formalin:  $F_{(1,41)}=18.43$ , p<0.01, strain x formalin  $F_{(1,41)}=0.31$ , p=0.58; Distance moved: strain;  $F_{(1,44)}=39.05$ , p<0.01, formalin;  $F_{(1,44)}=44.83$ , p<0.01, strain x formalin  $F_{(1,44)}=10.31$ , p<0.01; Duration of freezing strain  $F_{(1,41)}=0.09$ , p=0.92, formalin  $F_{(1,41)}=5.32$ , p<0.05, strain x formalin  $F_{(1,41)}=1.49$ , p=0.23; \*p<0.05, \*\*p<0.01 vs. SD-Sal, +p<0.05, ++p<0.01 vs. WKY-Sal, and ##p<0.05 vs. SD-Form (ANOVA followed by Fisher's LSD post-hoc test); Data are mean±SEM (n=12) SD, Sprague- Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin

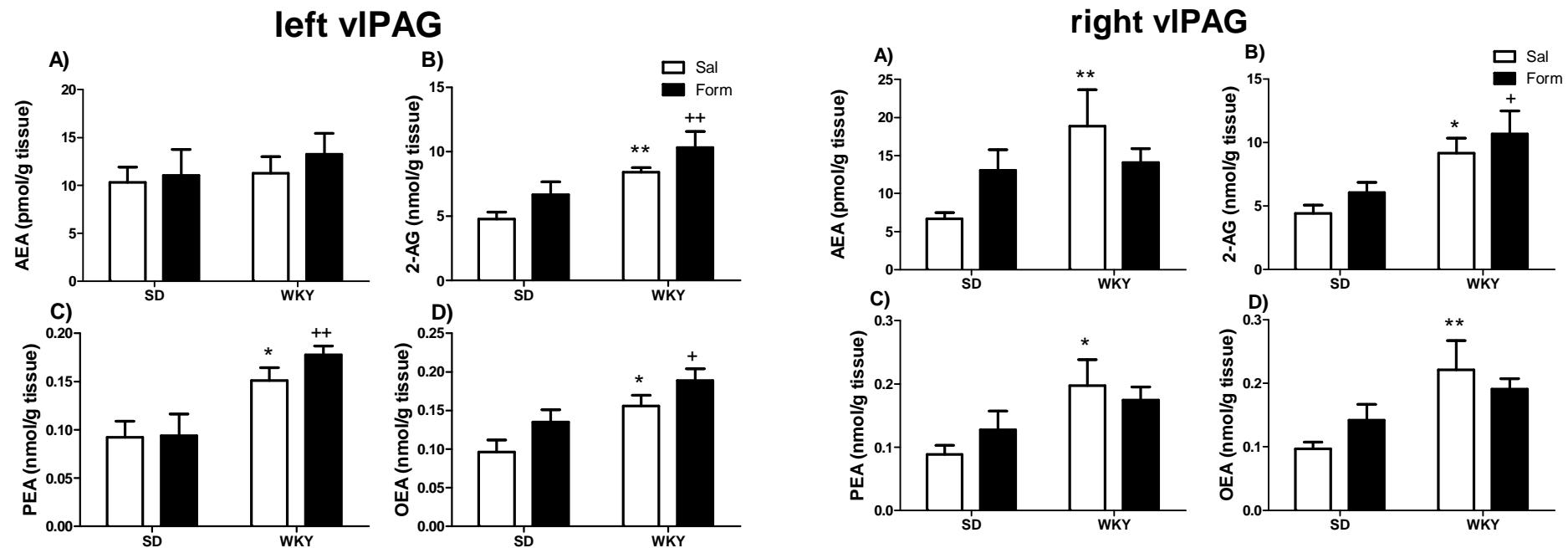
### **5.3.4 Levels of endocannabinoids and NAEs in discrete brain regions of SD and WKY rats receiving intra-plantar injection of saline or formalin**

Data for all analytes and brain regions are presented in Table 5.3. In addition, datasets where significant changes in levels of endocannabinoids (i.e. AEA or 2-AG) were observed are also presented as histograms. In saline-injected rats, levels of endocannabinoids (AEA and 2-AG) and NAEs (PEA and OEA) were higher in the vIPAG of WKY rats than that of SD counterparts, except for AEA in the left vIPAG (SD-Sal vs. WKY-Sal, p<0.05, **Fig 5.5 & 5.6**). In the IPAG, 2-AG levels were increased in saline-injected WKY rats compared to SD controls (SD-Sal vs. WKY-Sal, p<0.05, **Fig 5.7 & 5.8**). Levels of PEA and OEA were higher in the RVM of WKY rats receiving saline injection than that of SD controls (SD-Sal vs. WKY-Sal, p<0.05, **Fig 5.10**). Levels of endocannabinoids and NAEs were not different in the BLA, dIPAG, vHipp or insular cortex of saline-injected SD and WKY rats, however, WKY rats had lower levels of 2-AG in the left dHipp and lower levels of OEA in the mPFC (SD-Sal vs. WKY-Sal, **Table 5.3**).

Formalin injection did not affect levels of endocannabinoids or NAEs in the dIPAG or vIPAG, though some clear trends towards decreased and increased levels of all analytes were seen in the right dIPAG and right vIPAG, respectively compared with saline-injected SD rats (SD-Sal vs. SD-Form, **Fig 5.6 & 5.9**). In the right IPAG of SD rats, intra-plantar formalin resulted in a significant decrease in levels of AEA, OEA and PEA, but not 2-AG (**Fig 5.8**). In comparison, in the RVM, levels of all analytes except AEA, were increased compared to saline-injected controls (SD-Sal vs. SD-Form, **Fig 5.10**). No statistically significant difference in levels of analytes in the BLA was found between saline- and formalin-injected SD rats despite a trend towards increased levels in formalin-treated rats (SD-Sal vs. SD-Form, **Table 5.3**). Intra-plantar injection of formalin to SD rats increased levels of 2-AG in the left vHipp and decreased levels of PEA and OEA in the mPFC compared to saline-treated SD rats, without affecting levels in other regions such as the insular cortex and dHipp (SD-Sal vs. SD-Form, **Table 5.3**).

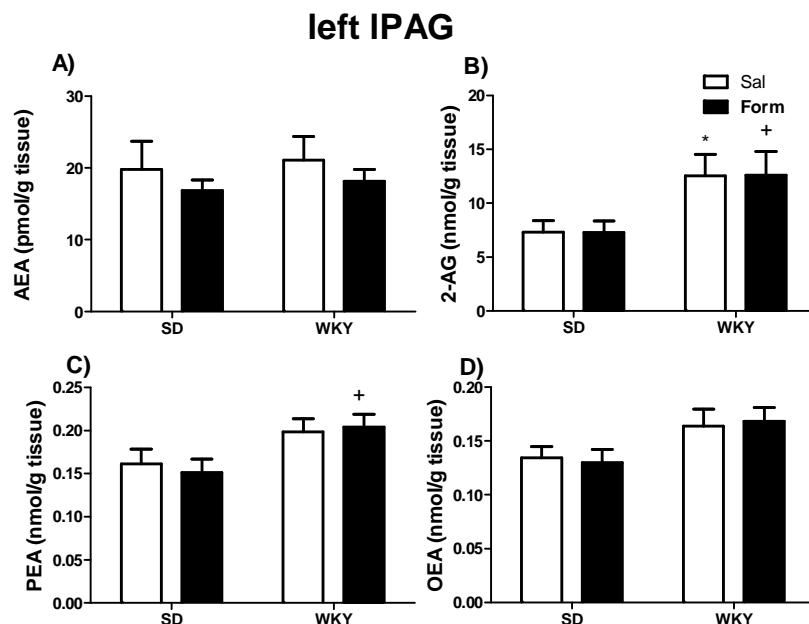
In WKY rats, intra-plantar formalin resulted in decreased levels of OEA and PEA in the RVM (Fig 5.10) and increased levels of all analytes in the right vHipp without affecting levels in the different sub-regions of the PAG, BLA, dHipp, insular cortex and mPFC (SD-Sal vs. SD-Form, **Table 5.3**).

Comparison between formalin-injected WKY and SD counterparts revealed increased levels of 2-AG, PEA and OEA in the left vlPAG with only 2-AG increased in the right vlPAG and right dlPAG and a strong trend towards increased levels of all analytes in the dlPAG of WKY rats (SD-Form vs. WKY-Form, **Fig 5.5, 5.6, 5.9 & Table 5.3**). Compared with formalin-treated SD counterparts, WKY rats receiving formalin had significantly higher levels of 2-AG and PEA in the left IPAG, higher levels of all analytes except AEA in the right IPAG, higher levels of PEA in the right BLA but lower levels of all analytes in RVM and 2-AG in left dHipp and vHipp when compared with SD counterparts (WKY-Form vs. SD-form,  $p<0.05$ , **Table 5.3**).

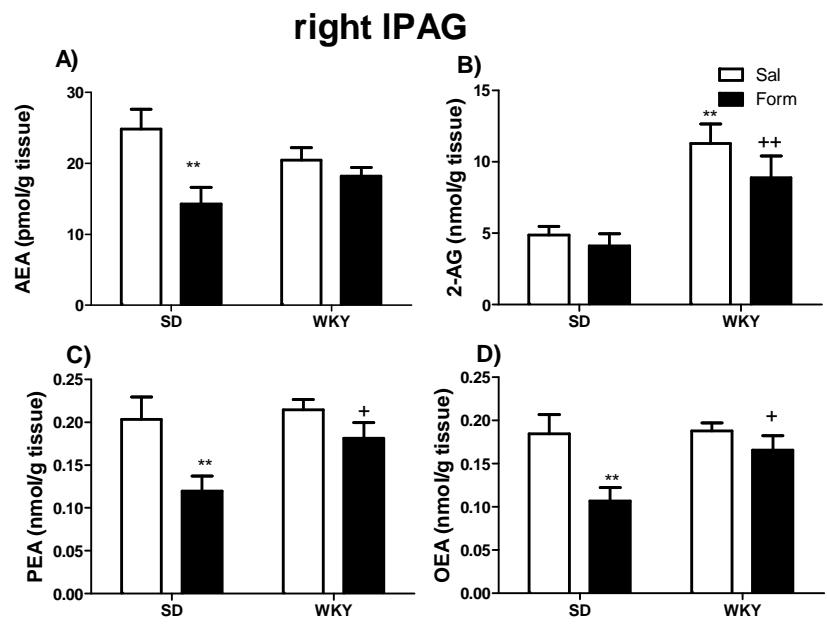


**Figure 5.5** AEA, 2-AG, PEA and OEA levels (A-D) in the left vIPAG following intra-plantar saline or formalin injection in SD and WKY rats A) AEA B) 2-AG C) PEA and D) OEA \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Sal, + $p<0.05$ , ++ $p<0.01$  vs. SD-Form, (Two-Way ANOVA followed by Fisher's LSD when appropriate); Data expressed as Mean  $\pm$  SEM (n=5-6). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

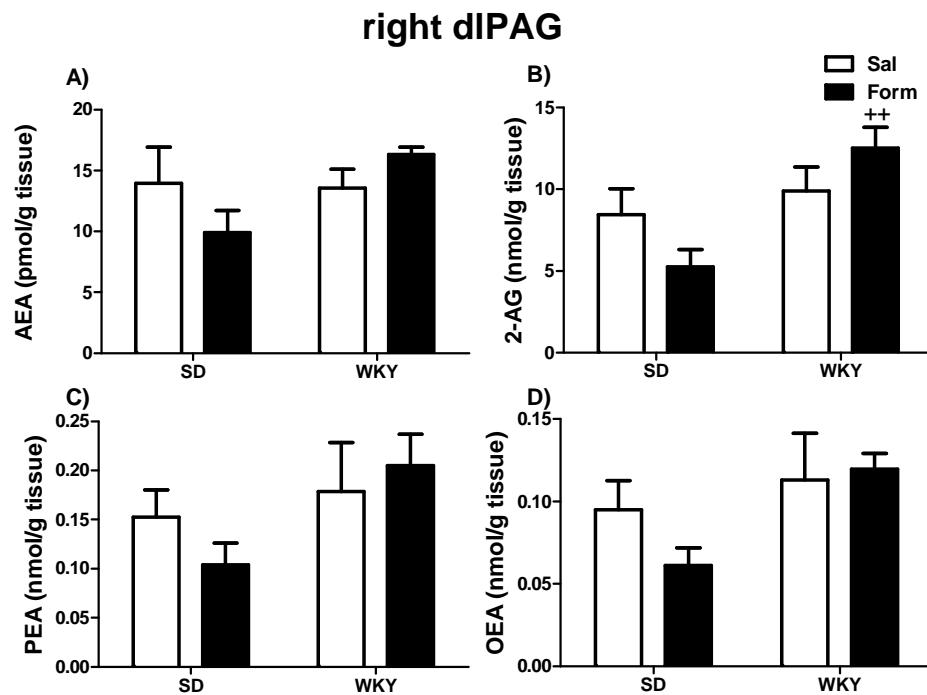
**Figure 5.6** AEA, 2-AG, PEA and OEA levels (A-D) in the right vIPAG following intra-plantar saline or formalin injection in SD and WKY rats A) AEA B) 2-AG C) PEA D) OEA \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Sal, + $p<0.05$  vs. SD-Form, (Two Way ANOVA followed by Fisher's LSD when appropriate); Data expressed as Mean  $\pm$  SEM (n=5-6). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide



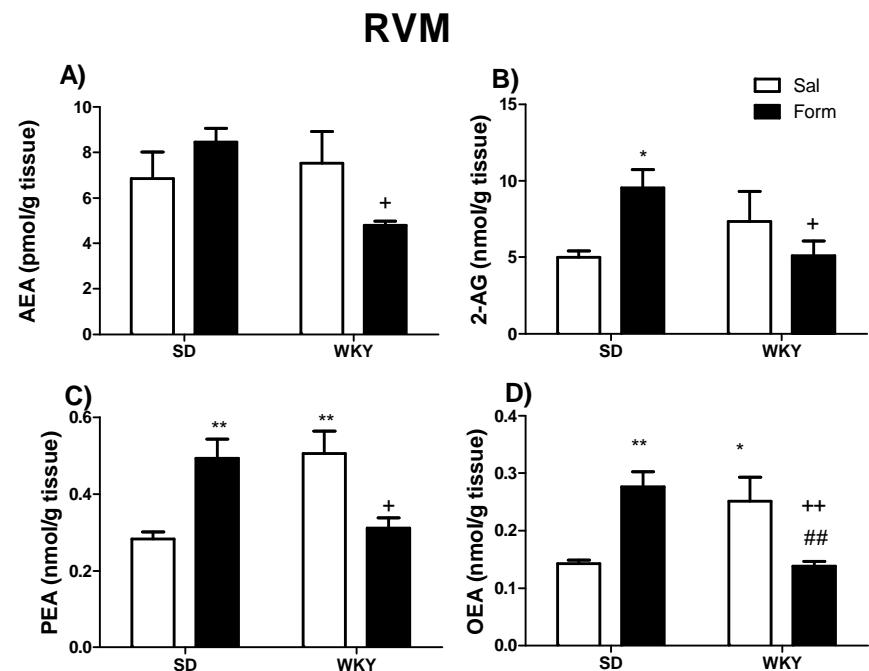
**Figure 5.7** AEA, 2-AG, PEA and OEA levels (A-D) in the left IPAG following intra-plantar saline or formalin injection in SD and WKY rats A) AEA B) 2-AG C) PEA D) OEA \* $p<0.05$  vs. SD-Sal, + $p<0.05$  vs. SD-Form, (Two Way ANOVA followed by Fisher's LSD when appropriate); Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, *N*-palmitoyl ethanolamide; OEA, *N*-oleoyl ethanolamide.



**Figure 5.8** AEA, 2-AG, PEA and OEA levels (A-D) in the right IPAG following intra-plantar saline or formalin injection in SD and WKY rats A) AEA B) 2-AG C) PEA D) OEA \*\* $p<0.01$  vs. SD-Sal, + $p<0.05$ , ++ $p<0.01$  vs. SD-Form, (Two Way ANOVA followed by Fisher's LSD when appropriate); Data expressed as Mean  $\pm$  SEM ( $n=5-6$ ). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, *N*-palmitoyl ethanolamide; OEA, *N*-oleoyl ethanolamide



**Figure 5.9** AEA, 2-AG, PEA and OEA levels (A-D) in the right dlPAG following intra-plantar saline or formalin in SD and WKY rats A) AEA B) 2-AG C) PEA D) OEA \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Sal, ++ $p<0.01$  vs. SD-Form (Two Way ANOVA followed by Fisher's LSD when appropriate); Data expressed as Mean  $\pm$  SEM (n=5-6). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.



**Figure 5.10** AEA, 2-AG, PEA and OEA levels (A-D) in the RVM following intra-plantar saline or formalin in SD and WKY rats A) AEA B) 2-AG C) PEA D) OEA \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Sal, + $p<0.05$ , ++ $p<0.01$  vs. SD-Form, #  $p<0.05$ , ## $p<0.01$  vs. WKY-Sal (Two Way ANOVA followed by Fisher's LSD when appropriate); Data expressed as Mean  $\pm$  SEM (n=5-6). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

**Table 5.3 Levels of AEA, 2-AG, PEA and OEA in discrete brain regions of SD and WKY rats in the presence or absence of formalin-induced nociception**

Region	Side	Analyte	SD-Sal	WKY-Sal	SD-Form	WKY-Form	Strain (F , DF, P values)	Form (F , DF, P values)	Interaction (F , DF, P values)
dlPAG	left	AEA	12.64 ±1.53	15.13 ±3.78	13.15 ±0.84	20.05 ±4.97	F 1,20= 2.10, 0.16	F 1,20=0.70,0.41	F 1,20=0.46,0.50
		2-AG	6.36 ±1.34	8.26 ±2.43	8.54 ±0.83	11.91 ±1.22	F 1,20=2.80,0.10	F 1,20=3.43, 0.08	F 1,20=0.22,0.65
		PEA	0.13 ±0.02	0.16 ±0.04	0.15 ±0.02	0.20 ±0.04	F 1,20=1.80,0.20	F 1,20=1.32,0.26	F 1,20=0.15,0.71
		OEA	0.086 ±0.01	0.09 ±0.02	0.099 ±0.01	0.127 ±0.02	F 1,20=1.03,0.32	F 1,20=1.82,0.19	F 1,20=37,0.55
	right	AEA	13.96 ±2.97	13.55 ±1.55	9.91 ±1.81	16.33 ±0.58	F 1,19= 2.21, 0.15	F 1,19=0.09,0.76	F 1,19=2.86,0.11
		2-AG	8.45 ±1.58	9.89 ±1.47	5.25 ±1.06	12.51 ±1.27++	F 1,20=10.24,0.00	F 1,20=0.05,0.83	F 1,20=4.55,0.046
		PEA	0.15 ±0.03	0.18 ±0.05	0.10 ±0.02	0.33 ±0.13	F 1,19=3.29,0.08	F 1,19=0.09, 0.76	F 1,19=1.14, 0.29
		OEA	0.095 ±0.02	0.11 ±0.03	0.06 ±0.01	0.24 ±0.12	F 1,19=4.11,0.057	F 1,19=0.51,0.48	F 1,19=1.16,0.29
vIPAG	left	AEA	10.31 ±1.60	11.28 ±1.72	11.06 ±2.72	13.26 ±2.19	F 1,18=0.2,0.66	F 1,18=0.91,0.352	F 1,18=0.36,0.56
		2-AG	4.78 ±0.53	8.42 ±0.35**	6.68 ±1.00	10.32 ±1.25++	F 1,20=6.90,0.016	F 1,20=1.12,0.30	F 1,20=0.04,0.84
		PEA	0.09 ±0.02	0.15 ±0.01*	0.09 ±0.02	0.18 ±0.01++	F 1,18=19.44,0.000	F 1,18=0.76,0.39	F 1,20=0.60,0.45
		OEA	0.11 ±0.01	0.156 ±0.01*	0.135 ±0.01	0.188 ±0.01+	F 1,18=14.17,0.002	F 1,20=5.65,0.03	F 1,20=0.04,0.85
	right	AEA	6.70 ±0.79	18.86 ±4.77**	13.07 ±2.71	14.09 ±1.82	F 1,20=5.11,0.035	F 1,20=0.008,0.79	F 1,20=3.64,0.07
		2-AG	4.42 ±0.66	9.15 ±1.19*	6.07 ±0.8	10.68 ±1.82	F 1,20=15.09,0.001	F 1,20=1.74,0.20	F 1,20=0.00,0.96
		PEA	0.077 ±0.01	0.197 ±0.04*	0.127 ±0.03	0.17 ±0.02	F 1,20=7.73,0.012	F 1,20=0.08,0.78	F 1,20=1.20,0.29
		OEA	0.097 ±0.00	0.22 ±0.02**	0.14 ±0.01	0.19 ±0.01	F 1,20=9.47,0.006	F 1,20=0.07,0.80	F 1,20=1.05,0.19
lateral PAG	left	AEA	19.78 ±3.93	21.12 ±3.30	16.87 ±1.48	18.16 ±1.63	F 1,20=0.22,0.64	F 1,20=1.10,0.31	F 1,20=0.00, 0.99
		2-AG	7.33 ±1.07	12.54 ±2.01*	7.28 ±1.1	12.62 ±2.18+	F 1,20=9.97,0.05	F 1,20=0.00,0.99	F 1,20=0.00, 0.97
		PEA	0.16 ±0.02	0.198 ±0.02	0.15 ±0.02	0.20 ±0.01+	F 1,20=8.19,0.01	F 1,20=0.02,0.89	F 1,20=0.27,0.61
		OEA	0.13 ±0.01	0.16 ±0.02	0.129 ±0.01	0.168 ±0.01	F 1,20=6.99,0.016	F 1,20=0.00,0.99	F 1,20=0.13,0.72
	right	AEA	24.86 ±2.78	20.47 ±1.76	14.29 ±2.35**	18.22 ±1.21	F 1,20=0.01,0.91	F 1,20=9.22,0.007	F 1,20=3.88,0.06
		2-AG	4.87 ±0.63	11.28 ±1.38**	4.10 ±0.88	8.89 ±1.51++	F 1,20=23.37,0.00	F 1,20=1.85,0.18	F 1,20=0.49,0.49
		PEA	0.20 ±0.03	0.21 ±0.01	0.119 ±0.02**	0.18 ±0.02+	F 1,20=3.62,0.72	F 1,20=9.24,0.006	F 1,20=1.74, 0.20
		OEA	0.18 ±0.02	0.188 ±0.01	0.12 ±0.02**	0.17 ±0.02+	F 1,20=3.46, 0.077	F 1,20=9.06,0.007	F 1,20=2.75,0.11

**Table 5.3 Continued**

Region	Side	Analyte	SD-Sal	WKY-Sal	SD-Form	WKY-Form	Strain (F ,DF, P values)	Form(F , DF, P values)	Interaction (F , DF, P values)
VHipp	left	AEA	157.96 ±21.31	104.79 ±24.15	154.87 ±16.66	118.57 ±26.66	F 1,18= 3.89,0.064	F 1,18=0.06,0. 82	F 1,18=0.14,0.71
		2-AG	13.37 ±1.7	16.04 ±2.06	19.24 ±0.75*	14.56 ±1.16+	F 1,20=0.45,0.51	F 1,20=2.13,0.16	F 1,20=5.96,.024
		PEA	0.296 ±0.04	0.24 ±0.07	0.31 ±0.04	0.199 ±0.05	F 1,19=2.55,0.13	F 1,19=0.06,0.81	F 1,19=0.23,0.64
		OEA	0.367 ±0.03	0.29 ±0.08	0.37 ±0.04	0.30 ±0.08	F 1,19=1.27,0.27	F 1,19=0.01,0.92	F 1,19=0.01,0.92
	right	AEA	121.10 ±23.80	60.85 ±26.34	165.99 ±32.52	234.32 ±30.22##	F 1,18= 6.02,0.89	F 1,18=14.47,0.001	F 1,18=5.02,0.038
		2-AG	12.39 ±3.88	9.669 ±4.09	20.80 ±4.09	21.71 ±1.76#	F 1,19=0.06,0.81	F 1,19=7.48, 0.013	F 1,19=0.24,0.63
		PEA	0.28 ±0.07	0.148 ±0.06	0.38 ±0.07	0.36 ±0.04#	F 1,19=1.56,0.23	F 1,19=5.82,0.026	F 1,19=0.71,0.41
		OEA	0.35 ±0.09	0.21 ±0.09	0.48 ±0.1	0.46 ±0.03#	F 1,19=0.91,0.35	F 1,19=4.97,0.038	F 1,19=0.46,0.50
DHipp	left	AEA	69.597 ±17.13	178.55 ±81.35	134.81 ±81.42	90.68 ±66.22	F 1,20=0.23,0.63	F 1,20=0.03,0.87	F 1,20=1.32,0.27
		2-AG	7.63 ±1.22	4.66 ±0.67*	7.54 ±1.07	4.98 ±0.44+	F 1,20=9.35,0.006	F 1,20=0.02,0.90	F 1,20=0.05,0.83
		PEA	0.26 ±0.05	0.33 ±0.09	0.23 ±0.06	0.25 ±0.11	F 1,18=0.35,0.56	F 1,18=0.43,.52	F 1,18=0.10,0.75
		OEA	0.18 ±0.03	0.21 ±0.05	0.27 ±0.11	0.19 ±0.08	F 1,20=0.09,0.76	F 1,20=0.18,0.62	F 1,20=0.44,0.52
	right	AEA	16.19 ±1.32	40.25 ±17.11	17.49 ±3.42	11.84 ±2.59	F 1,18=1.30,0.27	F 1,18=2.82,0.11	F 1,18=3.39,0.08
		2-AG	7.92 ±0.77	5.64 ±1.05	6.22 ±0.82	4.91 ±0.57	F 1,19=4.53,0.047	F 1,19=2.08,0.17	F 1,19=0.33,0.57
		PEA	0.31 ±0.02	0.579 ±0.3	0.25 ±0.05	0.16 ±0.02	F 1,18=1.65,0.22	F 1,18=3.64,0.073	F 1,18=0.59,0.45
		OEA	0.20 ±0.02	0.25 ±0.07	0.19 ±0.03	0.12 ±0.01	F 1,19=0.13,0.72	F 1,19=3.18,0.09	F 1,19=2.29,0.15
Insular cortex	left	AEA	74.25 ±9.04	90.07 ±21.34	79.33 ±11.23	67.76 ±13.00	F 1,19=0.02,0.88	F 1,19=0.39,0.54	F 1,19=1.00,0.33
		2-AG	1.24 ±0.10	2.14 ±0.6	1.20 ±0.16	1.35 ±0.14	F 1,20=2.62,0.12	F 1,20=1.65,0.21	F 1,20=1.35,0.26
		PEA	0.10 ±0.01	0.11 ± 0.02	0.12±0.02	0.12 ±0.02	F 1,19=0.07,0.80	F 1,19=0.24,0.63	F 1,19=0.38,0.55
		OEA	0.11 ±0.03	0.12 ±0.04	0.13 ±0.04	0.11 ±0.03	F 1,20=0.16,0.69	F 1,20=0.22,0.64	F 1,20=0.73,0.40
	right	AEA	84.99 ±10.36	99.93 ±15.93	93.13 ±17.85	77.88 ±18.36	F 1,19=0.00,0.99	F 1,20=0.19,0.67	F 1,20=0.91,0.35
		2-AG	1.26 ±0.14	2.50 ±0.70	1.68 ±0.48	1.44 ±0.19	F 1,20=1.28,0.27	F 1,20=0.53,0.48	F 1,20=2.84,0.12
		PEA	0.13 ±0.01	0.147 ±0.02	0.15 ±0.03	0.12 ± 0.02	F 1,19=0.01,0.92	F 1,19=0.04,0.84	F 1,19=1.36,0.26
		OEA	0.14±0.02	0.16 ±0.03	0.16 ±0.05	0.13 ±0.04	F 1,19=0.02,0.88	F 1,19=0.09, 0.77	F 1,19=2.19,0.15
PFC	AEA	48.22 ±10.46	32.04 ±2.77	29.42 ±2.59	32.17 ±3.58	F 1,20=1.32,0.27	F 1,20=2.55,0.13	F 1,20=2.62,0.12	
	2-AG	1.07 ±0.14	1.32 ±0.15	1.037 ±0.10	0.90 ±0.09	F 1,20=0.20,0.66	F 1,20=3.37,0.08	F 1,20=2.45,0.13	
	PEA	0.09 ±0.02	0.079 ±0.01	0.06 ±0.01*	0.06 ±0.01	F 1,19=0.39,0.54	F 1,19=6.36,0.02	F 1,19=0.36,0.56	
	OEA	0.12 ±0.06	0.08 ±0.01*	0.067 ±0.01**	0.077 ±0.01	F 1,20=1.39,0.25	F 1,20=5.13,0.035	F 1,20=3.69,0.069	

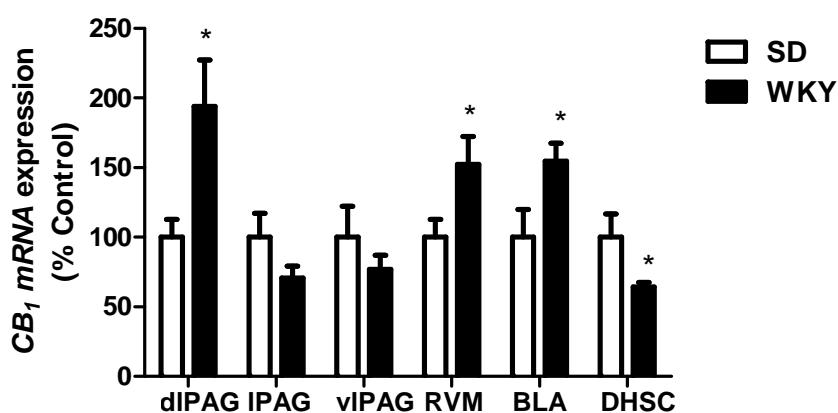
**Table 5.3 Continued**

Region	Side	Analyte	SD-Sal	WKY-Sal	SD-Form	WKY-Form	Strain(F , DF, P values)	Form(F , DF, P values)	Interaction (F , DF, P values)
BLA	left	AEA	22.99 ±3.04	14.92 ±2.51	30.55 ±3.30	18.87 ±2.73	F 1,20=2.0,0.17	F 1,20=0.42,0.52	F 1,20=2.23,0.15
		2-AG	3.38 ±0.44	4.07±0.44	3.49 ±0.69	4.32 ±0.62	F 1,20=1.83,0.19	F 1,20=0.11,0.75	F 1,20=0.02,0.89
		PEA	0.20 ±0.03	0.26 ±0.05	0.22 ±0.05	0.22 ±0.02	F 1,20=0.74,0.40	F 1,20=0.09,0.77	F 1,20=0.67,0.42
		OEA	0.11 ±0.01	0.139 ±0.02	0.138 ±0.02	0.12 ±0.01	F 1,20=0.1,0.76	F 1,20=0.07, 0.79	F 1,20=1.59,0.22
	right	AEA	11.83 ±3.11	25.08 ±7.06	19.84 ±3.24	22.75 ±5.91	F 1,19=2.53,0.13	F 1,19=0.31,0.58	F 1,19=1.04,0.32
		2-AG	2.62 ±0.66	3.70 ±0.23	3.40 ±0.62	4.24 ±0.45+	F 1,19=3.14,0.09	F 1,19=1.48,0.24	F 1,19=0.049,0.83
		PEA	0.167 ±0.03	0.218 ±0.02	0.20 ±0.03	0.34 ±0.08+	F 1,20=4.38,0.049	F 1,20=3.04,0.09	F 1,20=0.95,0.34
		OEA	0.089 ±0.02	0.12 ±0.01	0.12 ±0.02	0.14 ±0.02	F 1,19=2.87,0.11	F 1,19=1.55,0.23	F 1,19=0.09,0.77
RVM	AEA	6.86 ±1.16	7.54 ±1.39	8.46 ±0.6	4.79 ±0.18+	F 1,18=2.43,0.14	F 1,18=0.35,0.56	F 1,20=5.15,0.037	
	2-AG	4.99 ±0.42	7.34 ±1.98	9.54 ±1.19*	5.11 ±0.96+	F 1,20=0.68, 0.42	F 1,20=0.83,0.37	F 1,20=7.15,0.015	
	PEA	0.28 ±0.02	0.51 ±0.06	0.49 ±0.05**	0.31 ±0.03+	F 1,19=0.21,0.65	F 1,19=0.03,0.86	F 1,19=21.75,0.00	
	OEA	0.14 ±0.01	0.25 ±0.04	0.27 ±0.03**	0.14 ±0.01++ ##	F 1,19=0.29,0.59	F 1,19=0.15,0.71	F 1,19=21.24,0.00	

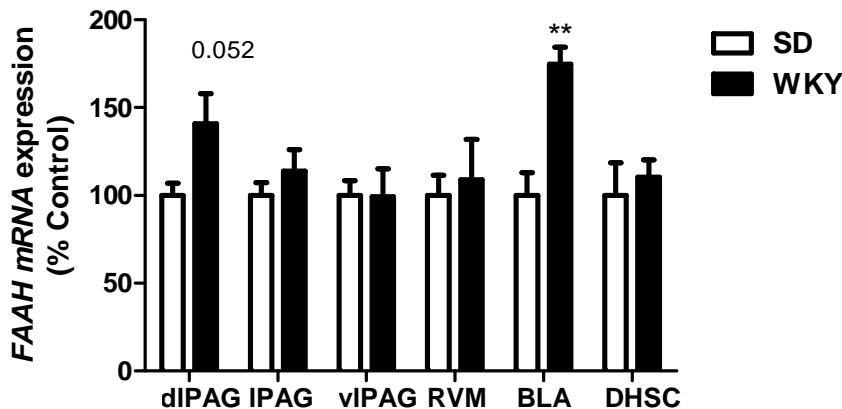
Levels of AEA, 2-AG, PEA and OEA in discrete brain regions of saline- or formalin-injected SD and WKY rats; \*p<0.05, \*\*p<0.01 vs. SD-Sal, +p<0.05, ++p<0.01 vs SD-Form, #p<0.01, ##p<0.05 vs. WKY-Sal (ANOVA followed by Fisher's LSD posthoc test); Data expressed as Mean pmol/g tissue ± SEM for AEA and Mean nmol/g tissue ± SEM for the other analytes (n=5-6). SD, Sprague-Dawley; WKY, Wistar-Kyoto; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

### **5.3.5 The expression of *CB<sub>1</sub>*, *FAAH* and *MAGL* mRNA in discrete brain regions and spinal cord of SD and WKY rats**

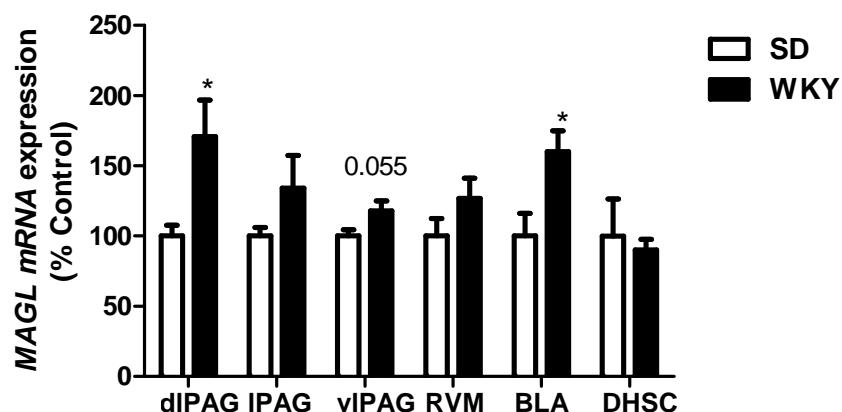
Expression of *CB<sub>1</sub>* mRNA in the dlPAG, RVM and BLA was higher in saline-treated WKY rats compared to SD counterparts (SD-Sal vs. WKY-Sal,  $p<0.05$ , **Fig 5.11**). However, levels of *CB<sub>1</sub>* mRNA were higher in the DHSC of SD rats than in WKY rats (SD-Sal vs. WKY-Sal,  $p<0.05$ , **Fig 5.11**). Levels of mRNA for the catabolic enzymes *FAAH* and *MAGL* were higher in the dlPAG and BLA of saline-treated WKY rats when compared with SD controls (SD-Sal vs. WKY-Sal,  $p<0.05$ , **Fig 5.12 &5.13**), though the increase in levels of *FAAH* in the dlPAG just failed to reach statistical significance (SD-Sal vs. WKY-Sal,  $p=0.052$ ). In addition, a strong trend towards increased expression of *MAGL* mRNA in the vIPAG was seen in saline-treated WKY rats compared to SD controls, however, this result just failed to reach statistical significance (SD-Sal vs. WKY-Sal,  $p=0.055$ ). Comparison of the expression of *GAPDH* mRNA, which was used as an endogenous control/reference gene, revealed no differences between the two strains in all the brain regions investigated (data not shown).



**Figure 5.11** Expression of *CB<sub>1</sub>* mRNA in discrete brain regions and in the DHSC of saline-treated SD and WKY rats. dlPAG:  $t_9=2.83$ ,  $p<0.05$ ; IPAG:  $t_9=1.43$ ,  $p=0.19$ ; vIPAG:  $t_9=0.87$ ,  $p=0.40$ ; RVM:  $t_9=2.29$ ,  $p<0.05$ , BLA:  $t_9=2.31$ ,  $p<0.05$ , DHSC:  $t_9=2.31$ ,  $p<0.05$ ; unpaired t-test \*  $p<0.05$  vs. SD-Sal for each region; data expressed as mean % control (SD-Sal)  $\pm$  SEM (n=5-6) SD, Sprague-Dawley; WKY, Wistar-Kyoto



**Figure 5.12** Expression of *FAAH* mRNA in discrete brain regions and in the DHSC saline-treated SD and WKY rats. dIPAG:  $t_{10}=2.20$ ,  $p=0.052$ ; IPAG:  $t_{10}=0.93$ ,  $p=0.38$ ; vIPAG:  $t_{10}=0.04$ ,  $p=0.971$ ; RVM:  $t_{10}=0.35$ ,  $p=0.74$ , BLA:  $t_{10}=4.65$ ,  $p<0.05$ , DHSC:  $t_9=0.52$ ,  $p=0.61$ ; unpaired t-test \*\* $p<0.01$  vs. SD-Sal for each region; data expressed as mean % control (SD-Sal)  $\pm$  SEM ( $n=5-6$ ); SD, Sprague-Dawley; WKY, Wistar-Kyoto



**Figure 5.12** Expression of *MAGL* mRNA in discrete brain regions and in the DHSC of saline-treated SD and WKY rats. dIPAG:  $t_{10}=2.63$ ,  $p<0.05$ ; IPAG:  $t_{10}=1.41$ ,  $p=0.19$ ; vIPAG:  $t_{10}=2.17$ ,  $p=0.055$ ; RVM:  $t_{10}=1.43$ ,  $p=0.18$ , BLA:  $t_{10}=2.74$ ,  $p<0.05$ , DHSC:  $t_9=0.39$ ,  $p=0.70$ ; unpaired t-test \* $p<0.05$  vs. SD-Sal for each region; data expressed as mean % control (SD-Sal)  $\pm$  SEM ( $n=5-6$ ); SD, Sprague-Dawley; WKY, Wistar-Kyoto

#### **5.4 Discussion**

The present study demonstrated that WKY rats exhibited enhanced anxiety-related behaviour and showed enhanced nociceptive responding to acute and persistent noxious stimuli compared to SD rats. The two rat strains differed with respect to baseline levels of endocannabinoids, NAEs and expression of *CB<sub>1</sub>*, *FAAH* and *MAGL* mRNA in discrete brain regions involved in modulating emotional and pain processes and in the DHSC. In addition, formalin-evoked nociceptive behaviour and ARH were associated with differential alterations in endocannabinoids and NAE levels in discrete brain regions in SD and WKY rats.

WKY rats exhibited an anxiogenic behavioural phenotype exemplified by the lower % time spent in the open arms of the EPM and less time spent in the centre of the open field, compared with SD counterparts. These findings are in line with previous reports that WKY rats exhibit an anxiogenic profile in a number of anxiety tests including the open field and EPM tests (Gentsch *et al.*, 1987; Paré, 1994; Burk *et al.*; 2010). WKY rats showed decreased locomotor activity in the EPM (both in the OA and total) and in the OF (both in the center zone and total). Previous studies have also reported that WKY rats exhibit an overall decreased activity/hypolocomotion and few exploratory behaviors (Pare, 1993, 1994, 1996; Armario *et al.*, 1995). Even though WKY rats were hypoactive (as shown by decreased total distance on the open field and elevated plus maze) compared to SD rats, they still spent the majority of the trial in the less aversive part of the elevated plus maze (i.e. CA) and open field (i.e. centre zone) indicating anxiogenic phenotype. In addition, the most commonly used index of locomotor activity on the elevated plus maze, % entry to the closed arms, did not differ between the two strains. It is possible that decreased activity of WKY rats in the open arms and the center zone contributed to the decrease in total activity. Several lines of evidence suggest that endocannabinoids in the brain modulate emotional responses. For instance, the endocannabinoid system in the BLA and PAG is important in the modulation of emotional processes (Bortolato *et al.*, 2006; Connell *et al.*, 2006; Kathuria *et al.*, 2003; Lafenêtre *et al.*, 2007; Lisboa *et al.*, 2008; Moreira *et al.*, 2007; Patel *et al.*, 2006a; Patel *et al.*, 2009). We report here that the anxiety-related phenotype in WKY rats was accompanied by an increase in levels of all analytes in the vIPAG (except AEA

in left vIPAG) and increased levels of 2-AG in the IPAG. The study also showed increased levels of OEA and PEA with a strong trend towards increased levels of AEA and 2-AG in the RVM, a group of nuclei in the brainstem crucial in the emotional motor system (Vianna *et al.*, 2008). Previous work has demonstrated increased levels of 2-AG and AEA in the rat dIPAG following footshock stress (Hohmann *et al.*, 2005) and our work in chapter 2 also has shown a fear-related increase in endocannabinoid levels in the dIPAG, vIPAG and RVM. The PAG is key component of the defensive response and different sub-regions of the PAG are known to mediate different forms of defensive responses (Bandler *et al.*, 1994). Thus, the functional and anatomical heterogeneity of the PAG could be the reason for differential alteration of endocannabinoids and NAEs in the different sub-regions of the PAG of WKY versus SD rats.

Despite previous work showing increased AEA and 2-AG levels in the BLA during conditioned fear (Marsicano *et al.*, 2002) and increased amygdalar 2-AG levels following restraint stress (Patel *et al.*, 2005; Rademacher *et al.*, 2008), we did not find alterations in the BLA of WKY rats compared with SD counterparts. However, comparison between these studies is difficult due to methodological differences. In comparison, our studies demonstrate that levels of 2-AG and PEA were lower in the dHipp and mPFC, respectively, of WKY rats, brain regions known to be involved in endocannabinoid-mediated modulation of emotional processes (Campos *et al.*, 2010; de Oliveira Alvares *et al.*, 2010; Laviolette *et al.*, 2006; Serra *et al.*, 2007). This finding stands in agreement with past work where exposure of rats to chronic unpredictable stress produced a reduction in the tissue content of AEA or 2-AG in the PFC and hippocampus (Hill *et al.*, 2005; Hill *et al.*, 2008). Furthermore, it was reported that in the mPFC, AEA decreased following the 7th and 10th restraint stress exposure (Rademacher *et al.*, 2008). Our result suggests a possibility that the endocannabinoids/NAEs in cortical and sub-cortical structures are differentially altered in an animal model of altered emotional responding, in line with previous report (Hill *et al.*, 2008).

It is possible that differences in baseline levels of endocannabinoids and NAEs in the vIPAG, IPAG and RVM might be responsible for the difference in

baseline emotionality between the two strains. Previously, differences in the level of monoamines between SD and WKY rats were reported as a possible reason underpinning their differential responsiveness to stress (Burke et al., 2010). The increased levels of endocannabinoids/NAEs in discrete brain regions of WKY rats can be interpreted in a number of ways: 1) It could be a physiological compensatory mechanism to counteract the anxiety-related behaviour that is observed in the WKY rats and which may arise from having a genetically deficient endocannabinoid system function that rendered them anxious. 2) Cannabinoids are known to induce diverse responses on anxiety- and fear-related behaviours. Even though the predominant effect is to induce anxiolytic-like effects, high doses often can produce the opposite effect (Moreira et al., 2010). The increased endocannabinoids in the IPAG and vIPAG acting through CB<sub>1</sub> receptors might be mediating the anxiety-related behaviour in WKY rats. 3) The increased endocannabinoids, particularly AEA, acting through the non-cannabinoid targets in the PAG and RVM, such as TRPV1 receptors might mediate the anxiety-related behaviour in WKY rats. Indeed, it has been reported that cannabinoids could mediate anxiogenic behaviour through activation of TRPV1 receptors in the dlPAG (Campos et al., 2009; Terzian et al., 2009), PFC (Rubino et al., 2008b) and hippocampus (Santos et al., 2008). Elucidation of the exact mechanism requires further experimentation involving systemic and intra-cerebral administration of pharmacological agents which target CB<sub>1</sub>, TRPV1, MAGL or FAAH.

It should be noted that the brain regional differences in endocannabinoid/NAE levels in WKY vs. SD rats does not appear to be due to altered expression of the degrading enzymes FAAH and MAGL. Thus, there were no differences in levels of FAAH or MAGL mRNA in the IPAG, vIPAG or RVM of WKY and SD rats, despite differences in levels of endocannabinoids/NAEs in these regions. Moreover, although there was higher expression of *FAAH* and *MAGL* in the dlPAG and BLA of WKY rats, we did not find any baseline differences in endocannabinoid or NAE levels in these regions between the two strains. In addition, despite the strong trend for higher *MAGL* mRNA in the vIPAG of WKY rats, levels of 2-AG were higher in vIPAG of WKY rats, compared with SD controls. The increase in the expression of the degrading enzymes may not

be associated with increased activity of the enzymes. Assessment of the enzymatic activity in these brain regions would be revealing in this respect. It should be noted that we measured tissue levels of endocannabinoids and NAEs and this may not reflect the concentration which is active at the synapse. Our findings of increased levels of expression of *FAAH* and *MAGL* mRNA in the BLA and dlPAG are consistent with previous reports showing chronic stress-induced upregulation of *FAAH* levels in the hippocampus (Reich *et al.*, 2009) and increased *MAGL* mRNA expression in the PFC and thalamus of socially isolated rats (Robinson *et al.*, 2010). In contrast, *FAAH* mRNA expression was shown to be lower in PFC and the caudate putamen of socially isolated rats; whereas, maternal deprivation decreased *MAGL* mRNA and protein levels in the hippocampus (Suárez *et al.*, 2010). Thus, the expression of these degrading enzymes is altered differentially depending on factors such as the type of stress and brain region investigated. It should also be noted that the present study is measuring levels of these genes in a genetically stress susceptible strain as opposed to measuring alterations following exposure to environmental stress.

Increased expression of *CB<sub>1</sub>* mRNA in the dlPAG, BLA and RVM and decreased *CB<sub>1</sub>* mRNA in DHSC was observed in WKY versus SD rats in the present study. Previous studies have demonstrated that chronic stress induces upregulation of *CB<sub>1</sub>* receptors in the hippocampus of female rats (Reich *et al.*, 2009) and increased *CB<sub>1</sub>* receptor mRNA expression in the cortex and thalamus of socially isolated male rats (Robinson *et al.*, 2010). However, chronic stress also downregulated *CB<sub>1</sub>* receptor expression in the hippocampus of male rats (Hill *et al.*, 2005; Reich *et al.*, 2009) without affecting levels in the limbic forebrain (Hill *et al.*, 2005). Chronic restraint stress has been shown to induce upregulation of peripheral *CB<sub>1</sub>* receptors in the colon which has been proposed to be part of the protective function of the endocannabinoid system against stress-induced visceral hypersensitivity (Shen *et al.*, 2010). Moreover, a decrease in *CB<sub>1</sub>* expression in the DRG was shown following chronic water avoidance stress (Hong *et al.*, 2009). The authors suggested that increased endocannabinoid levels during stress may downregulate *CB<sub>1</sub>* receptor expression, contributing to visceral hyperalgesia (Hong *et al.*, 2009). Comparison of these studies with the present experiment is difficult as most of those studies induced

chronic stress before measuring levels of CB<sub>1</sub> receptor expression while in the current experiment CB<sub>1</sub> receptor expression was measured in an unstressed but naturally stress hyperresponsive strain. In contrast to the higher expression of *CB<sub>1</sub>* mRNA in the dlPAG, BLA and RVM of WKY rats, *CB<sub>1</sub>* mRNA in DHSC was lower in WKY rats. Whether the changes in CB<sub>1</sub> receptor expression levels observed in the spinal and supraspinal neural structures of WKY rats have a protective or facilitatory role on ARH will require further study.

In addition to the anxiogenic phenotype, the WKY rats displayed significant thermal hyperalgesia in the hot plate test and enhanced nociceptive behaviour during both the first and second phases of the formalin test. This is in keeping with previous reports where WKY rats showed thermal hyperalgesia on the hot plate test or increased formalin-evoked nociception (Burke *et al.*, 2010) and mechanical hypersensitivity (Taylor *et al.*, 2001) compared with SD rats. WKY rats also showed exacerbated mechanical allodynia following the induction of chronic constriction nerve injury compared with Wistar rats (Zeng *et al.*, 2008). However, our finding is in contrast with other reports where there was no change in the tail flick test (Burke *et al.*, 2010; Taylor *et al.*, 2001), mechanical (Burke *et al.*, 2010), and following formalin-induced noxious stimuli (Taylor *et al.*, 2001) compared with SD counterparts. Methodological differences such as the concentration of formalin (1.25% vs. 2.5%), for example, may account for the discrepancies between these studies (Taylor *et al.*, 2001). Thus, together with the anxiogenic phenotype, WKY rats demonstrated hyperalgesia- and may therefore represent a useful model of ARH. It should also be noted that locomotor activity assessed in both the 10min pre-formalin trial and the 30min trial period was lower in WKY rats, suggesting that the differences in formalin-evoked nociceptive behaviour between the two strains are unlikely to be due to overt changes in locomotor activity and are more likely related to alterations in nociceptive processing. The data support the contention that WKY rats may represent a genetic model of ARH which can be used to understand neurochemical and molecular mechanisms of this co-morbid clinical condition.

We hypothesised that the ARH in WKY rats could be due to the differential response of endocannabinoids in supraspinal structures that modulate

nociceptive responding in WKY and SD rats. The present study demonstrated that formalin-induced nociception in SD rats was associated with a decrease in all analytes (except 2-AG) in the right IPAG without affecting levels in the other PAG sub-regions, and concurrently an increase in all analytes (except AEA) in the RVM. In contrast, formalin injection in WKY rats did not change levels in the PAG but decreased levels of OEA and PEA in the RVM indicating a differential response of endocannabinoids and NAEs to formalin in these brain regions in the two strains. Comparison between formalin-treated SD and WKY rats showed higher levels of endocannabinoids and NAEs in most PAG sub-regions of WKY rats but these changes were also seen in saline-treated WKY rats, indicating that they are strain-related baseline differences rather than formalin-strain interaction effects. However, in the RVM, where WKY rats were shown to have higher levels of OEA and PEA at baseline, all 4 analytes were reduced significantly following formalin injection indicating a blunted response of endocannabinoids and NEAs to inflammatory pain stimulus in this region in WKY rats. Previously, in rodents, mechanical allodynia and thermal hyperalgesia following spinal nerve ligation were accompanied by increased levels of AEA and 2-AG in the PAG, RVM, dorsal raphe magnus and DRG (Mitrirattanakul *et al.*, 2006; Petrosino *et al.*, 2007). Extracellular AEA levels in the dorsal and lateral PAG were increased following formalin injection (Walker *et al.* 1999). However, direct comparisons between these earlier studies and the present study are difficult to make due to differences in the models used (spinal nerve ligation versus formalin test), dose of the formalin administered (4%, 150 $\mu$ l into both hind paws vs. 2.5%, 50 $\mu$ l into right hind paw), time-points and sub-regions assayed and method of analysis (microdialysis vs. tissue levels). These changes in endocannabinoids during noxious stimuli were suggested to be compensatory in nature. Thus, failure to release endocannabinoids/NAEs in the RVM during exposure to noxious stimuli, for example, could be one possible reason for enhanced nociception in WKY rats. Previously, Burke *et al.* (2010) suggested that a differential response of the monoamines in discrete brain regions of WKY rats in comparison to SD in response to formalin could be the reason for the differential nociceptive responses seen in the WKY and SD strains (Burke *et al.*, 2010). Our finding suggests a possible similar role for

endocannabinoids/NAEs; however, further work is needed to elucidate the precise role of endocannabinoids and NAEs in ARH.

In conclusion, anxiety-related behaviour and ARH observed in the stress sensitive WKY rats are accompanied by alterations in levels of endocannabinoids/NAEs in discrete brain regions involved in anxiety and nociceptive responding. In addition, the two strains differed with respect to baseline expression levels of *CB<sub>1</sub>*, *FAAH* and *MAGL* mRNA in the BLA, dIPAG, vIPAG, RVM and DHSC. It is possible that alterations in central endocannabinoid function may, at least in part, underlie the co-morbidity of anxiety and pain. The data support the contention that the WKY rat represents a genetic model of ARH which can be used to understand neurochemical and molecular mechanisms of this co-morbid clinical condition. These data advance our understanding of neurochemical alterations associated with pain, anxiety and ARH.

**Chapter 6: Effects of CB<sub>1</sub> receptor blockade or FAAH inhibition on anxiety-related hyperalgesia and expression of plasticity-related genes zif268 and sgk1**

### **6.1 Introduction**

ARH is a phenomenon where stress or anxiety enhance pain responding, and is observed in both rodents (for review see Imbe *et al.*, 2006) and humans (Rhudy *et al.*, 2000). Although widely recognised, the neurochemical and molecular mechanisms mediating ARH are poorly understood. The endocannabinoid system is involved in nociceptive and aversive processing. A large body of data suggests a role for the endocannabinoid system in modulation of both pain (see reviews Guindon *et al.*, 2009a; Hohmann *et al.*, 2006) and anxiety-related behaviours (see reviews Finn, 2010; Moreira *et al.*, 2008). However, few studies have investigated the role of the endocannabinoid system in ARH. Restraint stress-induced increases in visceromotor reflex and electromyogram response to colorectal distension in rats was attenuated by intra-peritoneal administration of the CB<sub>1</sub> receptor agonist, arachidonyl-2-chloro ethylamine (ACEA); whereas administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, rimonabant, further enhanced the stress-induced visceral hyperalgesia (Shen *et al.*, 2010). In the same experiment, stress induced an up-regulation of colon CB<sub>1</sub> receptors (Shen *et al.*, 2010). In another study, water avoidance-induced visceral hyperalgesia in rats was prevented by pre-treatment with the CB<sub>1</sub> receptor agonist, WIN 55,212-2 (Hong *et al.*, 2009). Furthermore, levels of AEA were increased while CB<sub>1</sub> receptor expression was decreased in the dorsal root ganglia of the stressed rats (Hong *et al.*, 2009). These results suggest that endocannabinoid signalling through CB<sub>1</sub> may play an important role in stress-induced visceral hyperalgesia. However, the role of the endocannabinoid system in ARH to somatic painful stimuli has not been investigated.

In Chapter 5, we characterised a model of trait anxiety-induced hyperalgesia using WKY rats, which demonstrated enhanced anxiety-related behaviour and nociceptive responding compared to SD rats. WKY rats had higher levels of ECs and NAEs, and higher expression of *CB1*, *FAAH* and *MAGL* mRNA in discrete brain regions. In addition, a differential response of endocannabinoids and NAEs in response to formalin was seen in the two strains, demonstrating that ARH is associated with alterations in the levels of endocannabinoids and

related lipids in discrete brain regions involved in anxiety and nociceptive responding. However, the exact role of the endocannabinoid system in ARH is still not known.

FAAH is the main enzyme responsible for the metabolism of several endogenous fatty acid amides, including AEA, PEA, and OEA (Cravatt *et al.*, 1996). FAAH inhibitors, such as cyclohexylcarbamic acid 3-carbamoyl biphenyl-3-yl ester (URB597) increase levels of AEA *in vivo* and *in vitro* (Fegley *et al.*, 2005; Hohmann *et al.*, 2005; Kathuria *et al.*, 2003; Lichtman *et al.*, 2004a). Several studies have demonstrated robust hypoalgesic phenotypes after pharmacological inhibition of FAAH by URB597 (Hasanein, 2009; Hasanein *et al.*, 2008; Jayamanne *et al.*, 2006; Kathuria *et al.*, 2003; Kinsey *et al.*, 2009; Naidu *et al.*, 2010; Russo *et al.*, 2007), see Table 6.1). Differential effects of URB597 on nociceptive responding have been reported depending on the dose of URB597 used (Jayamanne *et al.*, 2006, Hasanein *et al.*, 2009, Kinsey *et al.*, 2009, Russo *et al.*, 2007) and the nature of the pain (see Table 6.1). Previous studies have demonstrated that URB597 reduces nociceptive responding to both thermal and inflammatory stimuli in both Wistar (Kathuria *et al.*, 2003, Hasanein *et al.*, 2009) and SD (Jayamanne *et al.*, 2006, Hasanein *et al.*, 2008) rats.

AM251 is an analogue of rimonabant and a potent CB<sub>1</sub> receptor antagonist/inverse agonist (Hájos *et al.*, 2002; Haller *et al.*, 2004; Patel *et al.*, 2006a; Rodgers *et al.*, 2005). Systemic AM251 prevents the analgesic effects of cannabinoid receptor agonists (Guindon *et al.*, 2007a; Hama *et al.*, 2007; Kalbasi Anaraki *et al.*, 2008; Liang *et al.*, 2007; Liu *et al.*, 2006a), FAAH inhibitors such as URB597 (Hasanein *et al.*, 2008; Jayamanne *et al.*, 2006; Schuelert *et al.*, 2011), and endocannabinoid reuptake inhibitors such as AM404 (Borsani *et al.*, 2007; Hasanein, 2009; Mitchell *et al.*, 2007). Although AM251 alone does not affect nociceptive responding in various rodent pain models (Schuelert *et al.*, 2011, Liang *et al.*, 2007, Guindon *et al.*, 2007, Hama and Sagen, 2007, (Ahmed *et al.*, 2010; Maione *et al.*, 2007) (see Table 6.1), hyperalgesic effects have been observed in the formalin test (Maione *et al.*, 2007). The effect of AM251 has been examined in various models of thermal,

neuropathic and inflammatory pain using a wide range of different doses from 0.5mg/kg to 3 mg/kg and different strains including SD and Wistar rats. However, to-date, there are no published studies investigating the effects of URB597 or AM251 on nociceptive responding in WKY rats.

In addition to its effects on pain-related behaviour, the FAAH inhibitor URB597 has also been shown to reduce anxiety-related behaviour (Kathuria *et al.*, 2003; Patel and Hillard, 2006; Rubino *et al.*, 2008; Moreira *et al.*, 2008). Systemic administration of URB597 reduced anxiety-related behaviour in the rat elevated zero-maze and isolation-induced ultrasonic vocalisation tests (Kathuria *et al.*, 2003), the elevated plus-maze (Patel and Hillard, 2006; Naidu *et al.*, 2007; Hill *et al.*, 2007; Moise *et al.*, 2008), open field test (Moriera *et al.*, 2008) and light dark box test (Scherma *et al.*, 2008). However, differential modulation of anxiety-related behaviour at different doses of URB597 (Kathuria *et al.*, 2003; Naidu *et al.*, 2007; Scherma *et al.*, 2008), or no effect on anxiety-related behaviour (Naderi *et al.*, 2008), have also been observed following systemic administration of URB597. Dose-dependent effects of URB597 on anxiety-related behaviour have been observed in a number of rat strains including SD (Sherma *et al.*, 2008) and Wistar (Kathuria *et al.*, 2003) rats. Thus, URB597 can modulate pain- and anxiety-related behaviours in both SD and Wistar rats, results predominantly showing anxiolytic and hypoalgesic effects with some discrepancies depending on the experimental protocol.

In contrast, pharmacological blockade of the CB<sub>1</sub> receptor by AM251 increases anxiety-related behaviours in the elevated plus maze or open-field tests in mice (Haller *et al.*, 2004; Patel *et al.*, 2006a; Rodgers *et al.*, 2005) and rats (Sink *et al.*, 2010) (see Table 6.2). In comparison, studies have also shown no effect of systemic AM251 on anxiety-related phenotypes in mice (Micale *et al.*, 2008; Thiemann *et al.*, 2009) (see Table 6.2). Moreover, the effect of AM251 on anxiety-related behaviour was shown to be dose-dependent with higher doses usually resulting in an anxiogenic effect and lower doses without effect (Haller *et al.*, 2004; Patel *et al.*, 2006a; Rodgers *et al.*, 2005; Sink *et al.*, 2010). These anxiogenic effects of CB<sub>1</sub> receptor blockade suggest that activation of this receptor would decrease anxiety. Thus, systemic administration of AM251 in

rodents may elicit no effect on nociceptive responding but result in an anxiogenic phenotype depending on the dose administered.

Just as suppression of pain could be advantageous in highly stressful or dangerous situations where other defence behaviours must precede pain responses in order to ensure survival, facilitation of pain could promote recuperative behaviours during some forms of stress, and enhance vigilance in situations where threat is possible, but not imminent. While neural projections from the amygdala, PAG and RVM constitute key components of the descending inhibitory pain pathway (Basbaum *et al.*, 1984a; Behbehani, 1995; Heinricher *et al.*, 2009; Hopkins *et al.*, 1978; Oka *et al.*, 2008), the neural circuitry responsible for ARH remains largely unknown. It is now known that brainstem-spinal pathways, involving particularly the RVM, not only suppress nociception, but under some pathophysiological conditions concurrent facilitatory influence may predominate and lead to enhancement of sensory hyperexcitability (Pertovaara, 1998). In the RVM, two populations of neurons, ON-cells and OFF-cells have been identified that are differentially recruited by higher structures important in fear, illness and psychological stress, including the PAG and amygdala, to enhance or inhibit pain (Heinricher *et al.*, 2009).

*zif268* and *sgk1* represent plasticity-related genes involved in diverse neuronal function including nociception (Rygh *et al.*, 2006, Delander *et al.*, 1997, Otahara *et al.*, 2003, Rahman *et al.*, 2002, Geranton *et al.*, 2007) and anxiety/fear-related behaviours (Malkani *et al.*, 2000, Hall *et al.*, 2000, Malkani *et al.*, 2000, Ressler *et al.*, 2002, Rosen *et al.*, 1998, Lee *et al.*, 2007). Altered expression of these genes in the dorsal horn of the spinal cord and in supraspinal structures has been demonstrated following different forms of noxious stimuli (Rygh *et al.*, 2006, Geranton *et al.*, 2007, Pearse *et al.*, 2001, Wei *et al.*, 1999, Wei *et al.*, 2000). The results presented in Chapter 4 demonstrated that FCA was associated with suppression of formalin-evoked *zif268* expression (and a strong trend in a similar direction for *sgk1*) in the ipsilateral DHSC. In addition, pharmacological blockade of the CB<sub>1</sub> receptor attenuated FCA and prevented the fear-induced reduction of *zif268* expression in formalin-treated rats. Moreover, conditioned-fear was associated with increased expression of *zif268* in the RVM with a very

strong similar trend in the amygdala. However, the extent to which these genes are involved in stress/anxiety-related hyperalgesia is not known.

The aims of the experiments presented in this Chapter were:

- To investigate the role of the CB<sub>1</sub> receptor and endocannabinoids in ARH. This was achieved by examining the effect of systemic administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, AM251, or inhibitor of FAAH, URB597, on ARH using the model of trait ARH (WKY versus SD rats) that was described in Chapter 5. A sub-aim was to use responsivity to thermal stimulation (hot plate) as a means of selecting the most appropriate doses of AM251 and URB597 for administration prior to intra-plantar formalin injection.
- To determine if ARH, and its modulation by URB597/AM251, is associated with altered expression of *sgk1* and *zif268* in the amygdala, PAG and RVM.

The work tests the hypothesis that enhancing endocannabinoid signalling using URB597 attenuates ARH and reducing endocannabinoid signalling using AM251 enhances ARH in WKY rats.

**Table 6.1 Summary of studies investigating the effects of URB597 and AM251 on nociceptive responding in rodents**

	<b>species/strain</b>	<b>Model</b>	<b>Dose</b>	<b>Route</b>	<b>Volume</b>	<b>Time of injection</b>	<b>Effect of the drug</b>	<b>Reference</b>
<b>URB597</b>	Rat/SD	CFA/SNL	0.03,0.1,0.3	ip	1ml/kg	30min before -6hrs after drug injection	0.1&0.3mg/kg analgesic on CFA/no effect on SNL	Jayamanne et al., 2006
	Mice/ICR	LPS/HP	10mg/kg	ip	10µl/g	60min	analgesic	Naidu et al., 2010
	Rat/Wistar	HP	0.5mg/kg	ip		60min	analgesic	Kathuria et al., 2003
	Rat/Wistar	Formalin, TF, STZ	0.1,0.3,0.5mg/kg	ip			0.3&0.5mg/kg analgesic	Hasanein et al., 2009
	Rat/SD	TF	0.3mg/kg	ip	1ml/kg	60min	analgesic	Hasanein et al., 2008
	Mice/C57BL/6J	CCI	2-10mg/kg	ip	10µl/g	60min/90min	10mg/kg analgesic	Kinsey et al., 2009
	Mice/Swiss	CCI	1-50mg/kg	po		2 hrs	3-50mg/kg analgesic	Russo et al., 2007
	Rat/LH	formalin	0.3mg/kg	ip	1ml/kg	60min	no effect	Butler et al., 2008
	Rat/Wistar	OA	0.3,1,3,5mg/kg	sc	1ml/kg	60min	3&5mg decreased firing inhibited URB597 effect/ no effect alone	Schuelert et al., 2010
<b>AM251</b>	Rat/Wistar	OA	1mg/kg	ip	1ml/kg	30-60min		Schuelert et al., 2010
	Rat/SD	SCI	3mg/kg	sc	0.5ml	45 min	no effect	Ahmed et al., 2010
	Rat/SD	SCI	3mg/kg	sc	2 ml/kg	30 min	no effect alone/ attenuated effect of paracetamol	Hama and Sagen, 2009
	Rat/SD	TF	1mg/kg	ip	1ml/kg	30min	prevented effect of AM 404	Hasanein et al., 2009
	Rat/SD	TF	1mg/kg	ip	1ml/kg	10min	prevented effect of UCM707	Hasanein et al., 2008
	Rat/SD	TF	1mg/kg	ip	1ml/kg	60min	prevented effect of URB597	Hasanein et al., 2008
	Mice/	TF	0.5mg/kg	ip		30 min	prevented effect of WIN	Kalbasi et al., 2008
	Rat/SD	CFA/PNL	1mg/kg	ip	1ml/kg		prevented effect of AM404	Mitchell et al., 2007
	Rat/SD	CCI	1mg/kg	ip	1ml/kg	150min	no effect alone/prevented effect of WIN	Liang et al., 2007
	Rat/Wistar	SNL	3mg/kg	ip	1ml/kg	24hr	no effect alone/prevented effect of WIN	Guindon et al., 2007
	Rat/SD	Formalin	1mg/kg	ip		90 min	no effect alone/prevented effect of AM404	Borsari et al., 2007
	Rat/Wistar; Mice/	Formalin/CCI	3mg/kg	ip	1ml/kg	20min	slight hyperalgesic effect on formalin test <i>per se/</i> no effect <i>per se</i> in CCI	Maione et al., 2007
	Rat/SD	formalin	3mg/kg	sc	2ml/kg	60min	no effect alone/prevented WIN	Hama and Sagen, 2007
	Rat/SD	CCI	imkg/kg	iv	1ml/kg	tested every 5min after injection	prevented effect of WIN	Liu et al., 2006
	Rat/SD	CFA/SNL	1mg/kg	ip	1ml/kg	30min before -6hrs after drug injection	prevented URB597 induced analgesia	Jayamanne et al., 2006

LH, Lister hooded; SD, Sprague-Dawley; ip, intra-peritoneal; sc, subcutaneous; po, per os (mouth); iv, intra-venous; OA, monoiodoacetate; CFA, complete Freund's adjuvant; SNL, spinal nerve ligation; LPS, Lipopolysaccharide; HP, Hot plate; TF, Tail Flick; STZ, streptozotocin; CCI, chronic constriction injury; SCI, spinal cord injury; PNL, peripheral nerve ligation; Time of injection refers to time before behavioural test unless indicated.

**Table 6.2 Summary of studies investigating the effects of URB597 and AM251 on anxiety-related behaviour in rodents**

Anxiety	Species	Model	Dose	Route	Volume	Time of injection	Effect of the drug	Reference
URB597	Mice/C57BL/6N	EPM/LDT/OF	1mg/kg	ip	10ml/kg	2hr	anxiolytic	Moriera et al., 2008
	Rats/Wistar	EZM	0.05, 0.1mg/kg	ip		30min	0.1mg/kg anxiolytic	Kathuria et al.,2003
	Mice/ICR	EPM	0.1-0.3mg/kg	ip	10ml/kg	30min	0.1&0.3 anxiolytic	Patel&Hillard 2006
	Mice/ICR	EPM	0.1-10mg/kg	ip	10µl/g	30/120min	0.1mg/kg at 120min anxiolytic	Naidu et al.,2007
	Rats/SD	LDT	0.1&0.3mg/kg	ip	1ml/kg	40min	anxiolytic and anxiogenic with AEA	Scherma et al., 2008
	Mice/NMRI	EPM	0.03, 0.3mg/kg	ip	50µl	30min	no effect	Naderi et al.,2008
	Rats/LE	EPM/OF	0.1,0.3mg/kg	sc		1hr	anxiolytic	Hill et al., 2007
	Rats/SW	EPM	0.1-0.3mg/kg	ip	1ml/kg	30min	anxiolytic	Moise et al., 2008
	Mice /CD1	EPM	0.3,1,3mg/kg	ip	10ml/kg	30min	3mg/kg anxiogenic	Haller et al.,2004
	Mice/ Swiss-Webster	EPM	1.5-3mg/kg		10ml/kg	30min	3mg/kg anxiogenic	Roger et al.,2005
AM251	Mice/ ICR	EPM	1-10mg/kg	ip	10ml/kg	30min	3&10mg/kg anxiogenic	Patel and Hillard,2006
	Rats/Wistar;							
	Mice/CD1	EPM	0.3-3mg/kg	ip	10ml/kg	30min	no effect in rats/in mice 1&3 mg/kg anxiogenic	Haller et al., 2007
	Rats/SD	OF/EPM	2, 4 or 8mg/kg	ip	1ml/kg	30 for OF/2h for EPM	4 & 8mg/kg anxiogenic	Sink et al.,2010
	Mice/CD1	OF	3mg/kg	ip	5ml/kg	30min	no effect	Theimann et al., 2009
	Mice/C57BL/6J	EPM	1mg/kg	ip	10ml/kg	30min	no effect	Micale et al., 2009

LE, Long Evans; SH, Syrian Hamster; SD, Sprague Dawley; ip, intra-peritoneal; sc, subcutaneous; EPM, Elevated Plus Maze; OF, Open Field;  
Time of injection refers to time before behavioural test unless indicated.

## **6.2 Methods**

### **6.2.1 Animals**

Experiments were carried out on adult male SD (250-350g, on experimental days, n=32) and WKY (250-350g, on experimental days, n=32) rats (Harlan, UK) all singly housed and maintained at a constant temperature ( $21 \pm 2^{\circ}\text{C}$ ) under standard lighting conditions (12:12h light: dark, lights on from 07.00 to 19.00h). Rats were acclimatized to the animal unit for 7 days prior to experimentation. All experiments were carried out during the light phase between 08.00h and 17.00h. Food and water were available *ad libitum*. The experimental protocol was carried out following approval from the Animal Care and Research Ethics Committee, National University of Ireland, Galway, under license from the Department of Health and Children in the Republic of Ireland and in accordance with EU Directive 86/609.

### **6.2.2 Drug preparation**

The CB<sub>1</sub> receptor antagonist/inverse agonist AM251 (1-[2,4-dichlorophenyl]-5-[4-iodophenyl]-4-methyl-N-[piperidin-1-yl]-1H-pyrazole-3-carboxamide; (Ascent Scientific, Bristol, UK) and the FAAH inhibitor URB597 (30-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate; Sigma Aldrich, Dublin, Ireland) were prepared on day of use to concentrations of (1, 3 or 5mg/kg) and (0.1, 0.2 or 0.5mg/kg), respectively, in ethanol:cremophore:saline (1:1:18) and administered at an injection volume of 3ml/kg.

### **6.2.3 Behavioural testing**

#### **6.2.3.1 Experiment 1: Effects of AM251 on nociceptive responding to thermal stimulus and on anxiety-related behaviour**

Following 7 days of acclimatization to the facility, SD and WKY rats received intra-peritoneal injection of AM251 (1, 3 or 5mg/kg) or vehicle (ethanol:cremophore:saline; 1:1:18). 30min post administration rats were exposed to the hot plate, followed 5 minutes later by exposure to the elevated plus maze for a period of 5 minute and subsequently the open field for a further 5 minutes. Behaviour in the hot plate/open field/elevated plus maze tests was assessed as described in Chapter 5 (section 5.2.2). The choice of doses and time of administration was based on previous work (see Tables 6.1 and 6.2) and

included doses which have been shown to have an effect on pain and anxiety *per se* as well as on anxiety/fear-induced modulation of pain. This design resulted in eight experimental groups (n = 8 per group); SD-Veh, SD-AM251 (1mg/kg), SD-AM251 (3mg/kg), SD-AM251 (5mg/kg), WKY-Veh, WKY-AM251 (1mg/kg), WKY-AM251 (3mg/kg), WKY-AM251 (5mg/kg).

**6.2.3.2 Experiment 2: Effects of URB597 on nociceptive responding to a thermal stimulus and on anxiety-related behaviour**

Following a period of 1 week post-AM251 administration, the same group of rats received URB597 (0.1, 0.2 or 0.5mg/kg) or vehicle (ethanol:cremophore:saline; 1:1:18). 30min post administration rats were exposed to the hot plate, followed 5 minutes later by exposure to the elevated plus maze for a period of 5 minute and subsequently the open field for a further 5 minutes. Behaviour in the hot plate/open field/elevated plus maze tests was assessed as described in Chapter 5 (section 5.2.2). Rats were re-assigned to groups using a modified latin square design which ensured counterbalancing of treatments across the cohort of rats such that an equal number of rats in each treatment group received each possible combination of treatments/doses over the course of the two experiments. As with AM251, the choice of doses and time of administration for URB587 were based on previous work demonstrating efficacy on anxiety/fear, pain or FCA tests (see Table 6.1 and 6.2). This design resulted in eight experimental groups (n = 8 per group): SD-Veh, SD-URB597(0.1mg/kg), SD-URB597 (0.2mg/kg), SD-URB597 (0.5mg/kg), WKY-Veh, WKY-URB597 (0.1mg/kg), WKY-URB597 (0.2mg/kg), WKY-URB597 (0.5mg/kg).

**6.2.2.3 Experiment 3: Effects of AM251 and URB597 on formalin-induced nociceptive behaviour**

Nociceptive behaviour in the formalin test was assessed for each animal, at least 7 days following the administration of URB597. FAAH activity and AEA levels have been demonstrated to return to baseline 24hrs following single, acute, systemic administration of URB597 at these doses (Fegley *et al.*, 2005; Piomelli *et al.*, 2006). The procedure for this experiment was essentially as previously described in Chapter 5 (section 5.2.2) except that AM251 (3mg/kg), URB597

(0.5mg/kg) or vehicle were administered 30 and 60min prior to intra-plantar formalin injection, respectively. Rats were placed in a Perspex observation chamber (30x30x40 cm) for 10min prior to intra-plantar injection of 50µL formalin (2.5% in 0.9% saline) into the right hind paw under brief isoflurane anaesthesia as previously described (Finn *et al.*, 2003; Roche *et al.*, 2007, 2010; also described in previous Chapters 1-5). Rats were returned to their home cage for a further 3min at which point they were returned to the same Perspex observation chamber to which they had been previously exposed. Behaviour was recorded for 70 min from a video camera located beneath the observation chamber and rated as previously described.

In order to control for the different times of injection of the two drugs, AM251 and URB597, vehicle was administered at either 30 or 60min prior to intra-plantar formalin injection, resulting in two different vehicle groups for each strain. This design resulted in eight experimental groups (n = 6-10 per group): SD-Vehicle1 (30min) [SD-Veh1], SD-Vehicle2 (60min) [SD-Veh2], SD-AM251 (3mg/kg) [SD-AM251], SD-URB597 (0.5mg/kg) [SD-URB], WKY-Vehicle1 (30min) [WKY-Veh1], WKY-Vehicle2 (60min) [WKY-Veh2], WKY-AM251 (3mg/kg) [WKY-AM251] and WKY-URB597 (0.5mg/kg) [WKY-URB]. The two vehicle treatment groups of each strain were later combined as one group (SD-Veh1 and SD-Veh2 combined as SD-Veh; WKY-Veh1 and WKY-Veh2 combined as WKY-Veh) after statistical analysis confirmed no effect of the time of vehicle administration (i.e. 30 or 60 min) on any of the experimental parameters examined (SD-Veh1 vs. SD-Veh2 and WKY-Veh1 vs. WKY-Veh2), resulting in a total of 6 experimental groups (n = 10-12). The doses of AM251 and URB597 were selected based on the data from experiments 1&2, respectively, following analysis of anxiety-related behaviour hot plate test responding.

#### **6.2.4 Polymerase chain reaction (PCR)**

##### **6.2.4.1 RNA preparation**

Total RNA was extracted from homogenized tissue using a Machery-Nagel extraction kit (Nucleospin RNA II, Technopath, Dublin, Ireland) as described in Section 5.2 of Chapter 5.

#### **6.2.4.2 cDNA synthesis**

cDNA synthesis was carried out as described in Section 4.2.5.2 of Chapter 4.

#### **6.2.4.3 cDNA amplification**

The cDNA was used as the template for real-time quantitative PCR (RT-PCR), which was performed on the AB7500 PCR system (Applied Biosystems 7500) using TaqMan gene expression assays containing specific target primers and FAM-labelled probes (Applied Biosystems, Dublin, Ireland) with each gene-specific primer (see Table 5.1 for primer sequences). The procedure was carried out as described in Section 5.2.4 of Chapter 5.

**Table 6.3 Sequence of primers for RT-qPCR**

<b>Gene</b>	<b>Probe</b>	<b>Assay ID</b>	<b>Melting temperature (°C)</b>
GAPDH	VIC	4308313	63.4
Sgk1	FAM	Rn00561138_m1	64
Zif268	FAM	Rn00570285_m1	69

#### **6.2.5 Statistical analysis**

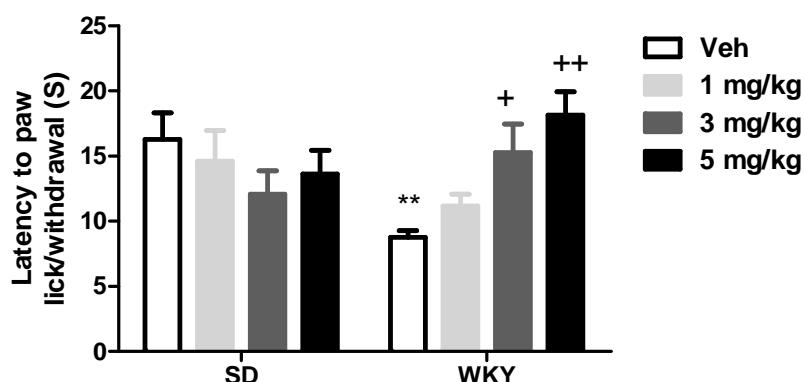
The SPSS 17.0 statistical package was used to analyse all data. Normality and homogeneity of data were assessed using Shapiro-Wilk and Levene test, respectively. Behavioural and molecular data were analysed using two-factor analysis of variance (ANOVA), with the factors being strain and drug. Post-hoc pair wise comparisons were made with Fisher's LSD when appropriate. Data are expressed as group means ± standard error of the mean (± SEM) and were considered significant when P<0.05.

### **6.3 Results**

#### **6.3.1 Experiment 1**

##### **6.3.1.1 Effect of AM251 on nociceptive responding to a thermal stimulus in WKY and SD rats**

Vehicle-treated WKY rats showed significant thermal hyperalgesia as measured by reduced latency to lick or withdraw either of their hind paws on the hot plate test when compared with vehicle-treated SD controls (SD-Veh vs. WKY-Veh,  $p<0.01$ , **Fig 6.1**). Intra-peritoneal administration of varying doses of AM251 did not affect the withdrawal latency of SD rats (SD-Veh vs. SD-AM251 (1, 3, 5mg/kg)); whereas, in WKY rats, AM251 produced a dose-dependent increase in latency to paw withdrawal, with the 3 and 5mg/kg doses resulting in a statistically significant increase in withdrawal latency when compared with the vehicle-treated controls (WKY-Veh vs. WKY-AM251 (3, 5mg/kg),  $p<0.05$ ).



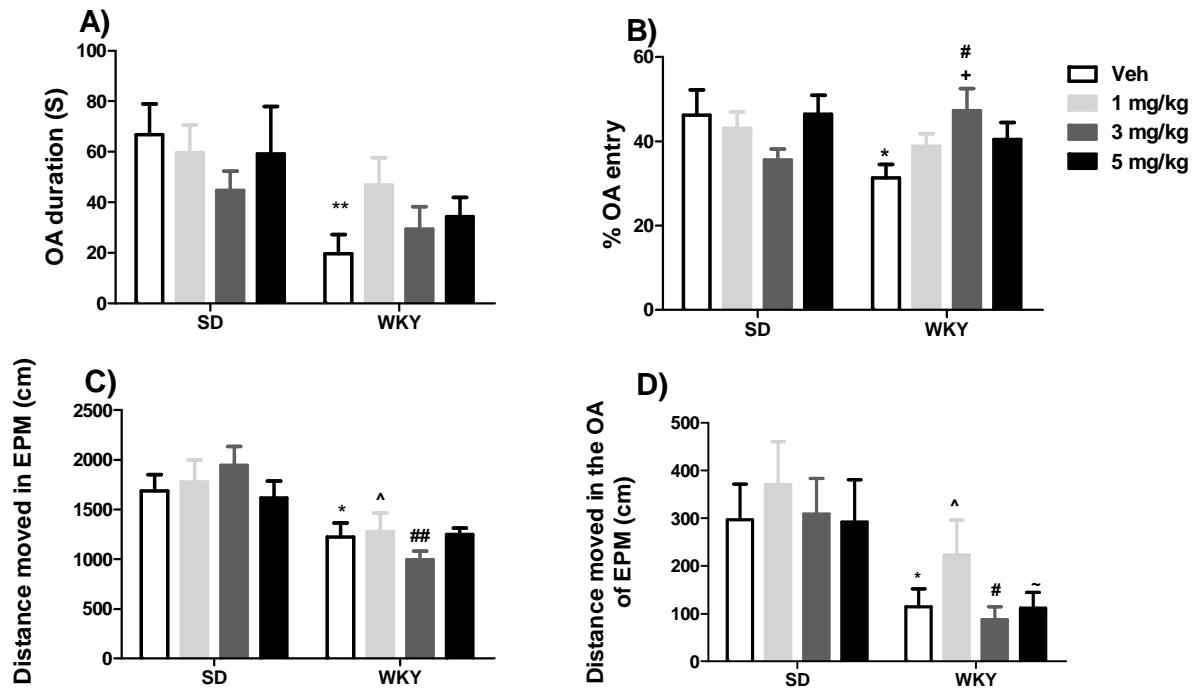
**Figure 6.1** Effect of increasing doses of AM251 on nociceptive responding to acute thermal stimulation on the hot plate test ( $55\pm1^{\circ}\text{C}$ ; (ANOVA: strain:  $F_{(1, 56)}=0.41$ ,  $p=0.52$ ; drug:  $F_{(3, 56)}=1.45$ ,  $p=0.23$  and strain x drug interaction:  $F_{(3, 56)}=5.09$ ,  $p<0.01$ ); \*\*  $P<0.01$  vs. SD-Veh, + $p<0.05$ , ++ $p<0.01$  vs. WKY-Veh; Data expressed as mean  $\pm$  SEM. (n=8); SD, Sprague-Dawley; WKY, Wistar-Kyoto.

##### **6.3.2.2 Effect of AM251 on anxiety-related behaviour in WKY and SD rats**

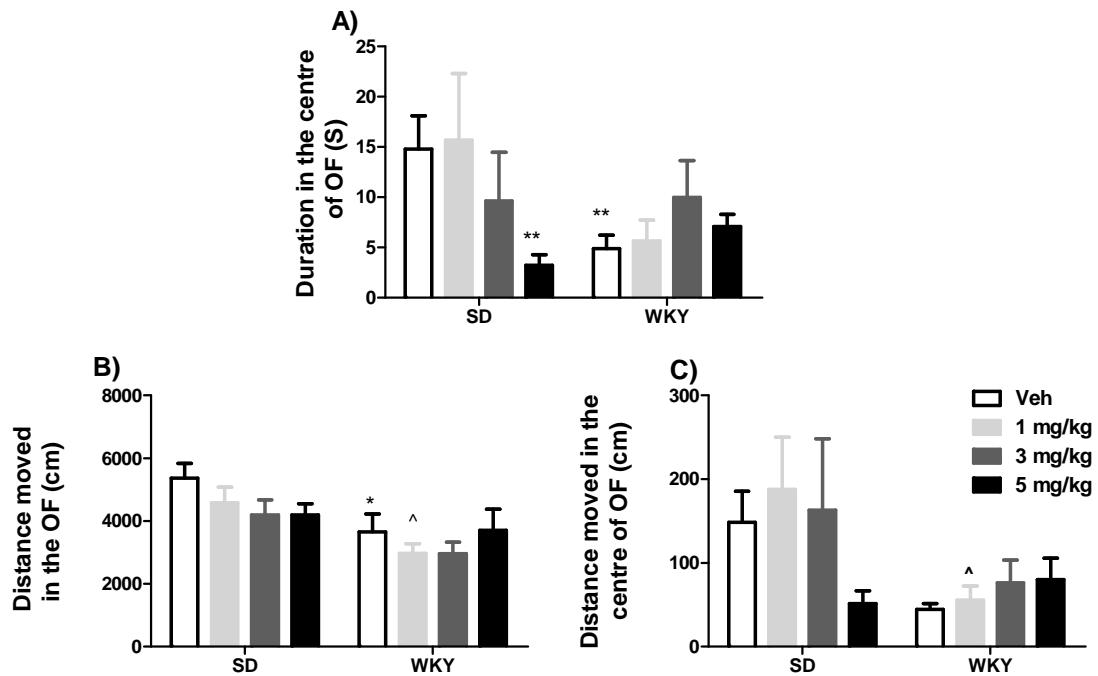
In the elevated plus maze test, data analysis revealed a significantly decreased time spent in the open arms and % entry into the open arms in vehicle-treated WKY rats during the 5 min trial period, compared with SD controls (SD-Veh vs. WKY-Veh,  $p<0.05$ , **Fig 6.2A&B**). In addition, vehicle-treated WKY rats

demonstrated decreased total distance moved and distance moved in the open arms, compared with vehicle-treated SD controls (SD-Veh vs. WKY-Veh, p<0.05, **Fig 6.2C&D**). No differences between the two strains were found for the numbers of entries into the closed arms, which is used as an index of general locomotion (% entry to closed arm of the elevated plus maze; SD-Veh, 53.72±5.91 vs. WKY-Veh, 56.21±5.72). In the open-field test, vehicle-treated WKY rats spent less time in the centre zone of the open-field when compared with SD counterparts indicating anxiety-like behaviour (SD-Veh vs. WKY-Veh, p<0.01, **Fig.6.3A**). Vehicle-treated WKY rats also exhibited decreased total distance moved and distance moved in the centre zone (SD-Veh vs. WKY-Veh, **Fig 6.3B&C**), though the reduction in distance moved in the centre zone did not reach statistical significance.

In SD rats, 3mg/kg AM251 decreased the time spent in the open arms of elevated plus maze (SD-Veh vs. SD-AM251(3mg/kg), **Fig 6.2A**) reaching statistical significance during the 4<sup>th</sup> and 5<sup>th</sup>min of the trial (data not shown) when compared with vehicle-treated controls. In contrast, in WKY rats, AM251 did not affect time spent in the open arms of the elevated plus maze at any of the doses used. In a similar manner, in SD rats, 3mg/kg AM251 decreased the % entry into the open arms when compared with vehicle-treated controls (SD-Veh vs. SD-AM251(3mg/kg), **Fig 6.2B**) though statistical significance was reached only during the 4<sup>th</sup> and 5<sup>th</sup>min (data not shown) of the trial. In contrast, systemic administration of 3mg/kg AM251 significantly increased the % entry into the open arm in WKY rats compared to the vehicle-treated controls (WKY-Veh vs. WKY-AM251 (3mg/kg), p<0.05, **Fig 6.2B**). In SD rats, 5mg/kg AM251 decreased the time spent in the centre of the open field compared to vehicle-treated SD rats (SD-Veh vs. SD-AM251 (5mg/kg), p<0.01, **Fig 6.3A**). However, AM251 did not affect the time spent in the centre of the open field in WKY rats at any of the doses tested. Locomotor activity (total distance moved) in the open field or in the elevated plus maze was not altered by AM251 in either SD or WKY rats at any of the doses examined when compared to vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-AM251, **Fig 6.2(C&D) and 6.3(B&C)**).



**Figure 6.2** Effect of AM251 on anxiety-related behaviours in SD and WKY rats, A) time spent in the open arms of the elevated plus maze (ANOVA: strain:  $F_{(1, 56)}=10.1$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.74$ ,  $p=0.53$  and strain x drug interaction:  $F_{(3, 56)}=0.98$ ,  $p=0.41$ ), B) % open arm entry (ANOVA: strain:  $F_{(1, 56)}=1.3$ ,  $p=0.26$ ; drugs:  $F_{(3, 56)}=0.42$ ,  $p=0.74$  and strain x drug interaction:  $F_{(3, 56)}=3.48$ ,  $p<0.05$ ), C) total distance moved in the EPM (ANOVA: strain:  $F_{(1, 56)}=24.97$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.13$ ,  $p=0.94$  and strain x drug interaction:  $F_{(3, 56)}=1.28$ ,  $p=0.29$ ), D) distance moved in the open arm of elevated plus maze (ANOVA: strain:  $F_{(1, 56)}=15.2$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=1.03$ ,  $p=0.39$  and strain x drug interaction:  $F_{(3, 56)}=0.12$ ,  $p=0.96$ ); \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Veh, # $p<0.05$ , ## $p<0.01$  vs. SD-AM251-3, + $p<0.05$  vs. WKY-Veh; ^ $p<0.05$  vs. SD-AM251-1 ~ $p<0.05$  vs. SD-AM251-5; Data are mean  $\pm$  SEM ( $n=8$ ), SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle; OA, open arms; EPM, elevated plus maze

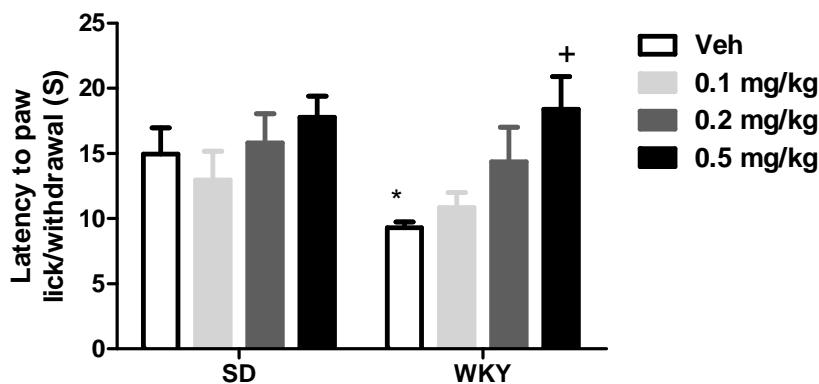


**Figure 6.3** Effect of AM251 on anxiety-related behaviours in SD and WKY rats, A) time spent in the centre zone of the open field (ANOVA: strain:  $F_{(1, 53)}=0.65$ ,  $p=0.42$ ; drugs:  $F_{(3,53)}=1.21$ ,  $p=0.31$  and strain x drug interaction:  $F_{(3,53)}=3.75$ ,  $p<0.05$ ), B) total distance moved in the open field (ANOVA: strain:  $F_{(1, 56)}=13.82$ ,  $p<0.01$ ; drugs:  $F_{(3,56)}=1.41$ ,  $p=0.25$  and strain x drug interaction:  $F_{(3,56)}=0.68$ ,  $p=0.56$ ), C) distance moved in the centre zone of the open field (ANOVA: strain:  $F_{(1, 56)}=5.96$ ,  $p<0.05$ ; drugs:  $F_{(3,56)}=0.75$ ,  $p=0.53$  and strain x drug interaction:  $F_{(3,56)}=1.38$ ,  $p=0.26$ ); \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Veh, ^ $p<0.05$  vs. SD-AM251-1; Data are mean  $\pm$  SEM ( $n=8$ ), SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle, OF, open field

### **6.3.2 Experiment 2**

#### **6.3.2.1 Effect of URB597 on nociceptive responding to thermal stimulus in WKY and SD rats**

Vehicle-treated WKY rats showed thermal hyperalgesia as measured by reduced latency to lick or withdraw either of their hind paws on the hot plate test when compared with the vehicle-treated SD controls (SD-Veh vs. WKY-Veh, **Fig 6.4**). Intra-peritoneal URB597 administration did not affect withdrawal latency of SD rats when compared to the vehicle-treated controls at any of the doses examined. However, in WKY rats, URB597 produced a dose-dependent increase in the withdrawal latency, with the 0.5mg/kg dose reaching statistical significance when compared with the vehicle-treated controls (WKY-Veh vs. WKY-URB597 (0.5mg/kg),  $p<0.05$ ).



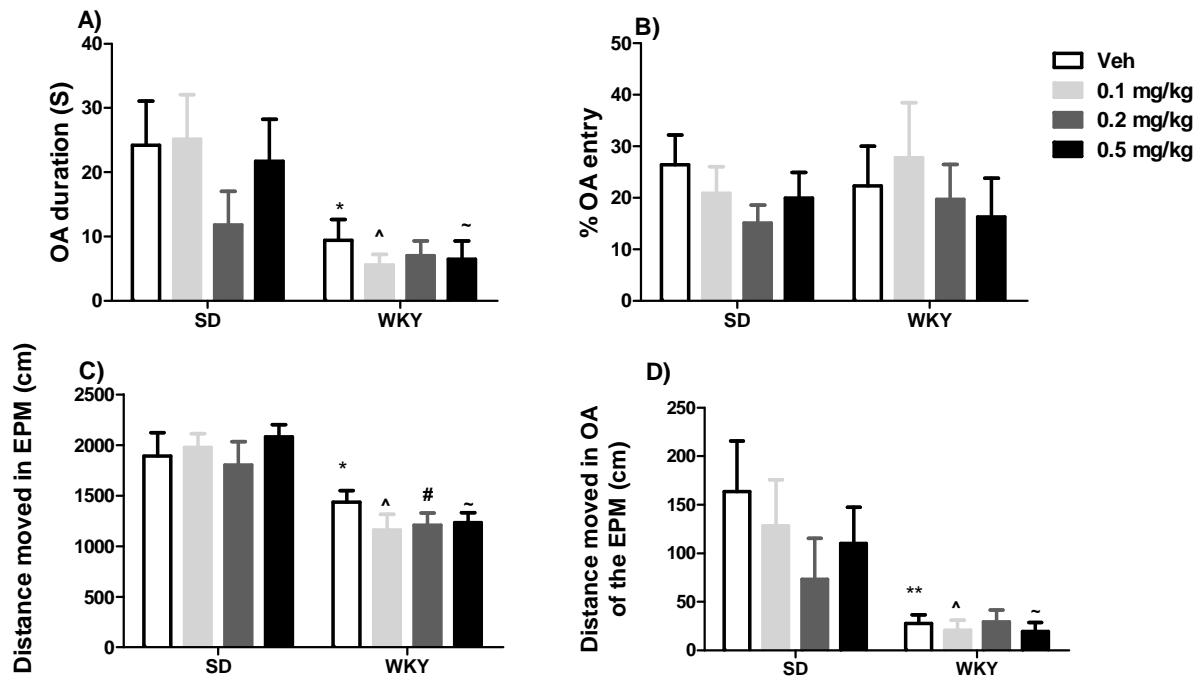
**Figure 6.4** Effect of increasing doses of URB597 on nociceptive responding to acute thermal stimulation on the hot plate test ( $55\pm1^{\circ}\text{C}$ ); (ANOVA: strain:  $F_{(1,56)}=2.35$ ,  $p=0.13$ ; drugs:  $F_{(3,56)}=4.3$ ,  $p<0.01$  and strain x drug interaction:  $F_{(3,56)}=0.87$ ,  $p=0.46$ ); \*  $P<0.05$  vs. SD-Veh, + $p<0.05$  vs. WKY-Veh.; Data expressed as mean $\pm$ SEM. (n=8); SD, Sprague-Dawley; WKY, Wistar-Kyoto

#### **6.3.2.2 Effect of URB597 on anxiety-related behaviour in WKY and SD rats**

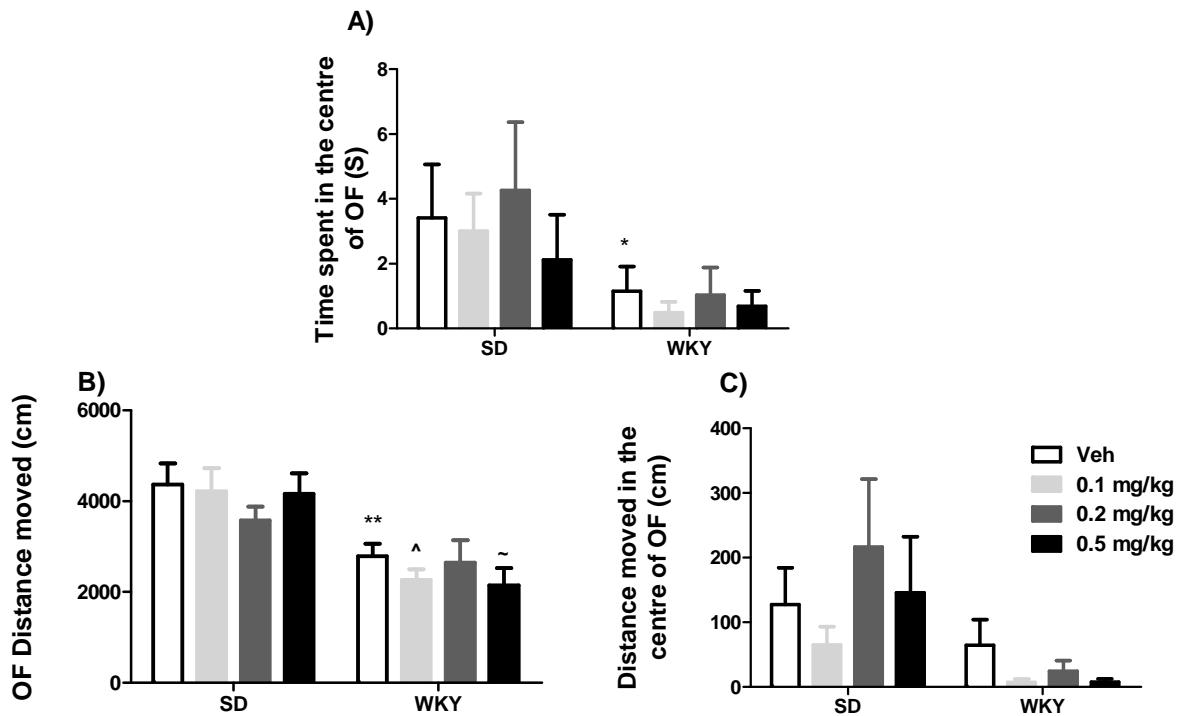
In the elevated plus maze test, vehicle-treated WKY rats spent significantly less time in the open arms compared to vehicle-treated SD controls (SD-Veh vs. WKY-Veh,  $p<0.05$ , **Fig 6.5A**). However, data analysis revealed no statistically significant difference in % entry into the open arms between vehicle-treated groups of the two strains over the total 5min trial (SD-Veh vs. WKY-Veh, **Fig.6.5B**). In addition, no difference was found in the numbers of entries into

the closed arms, which is used as index of general locomotion, between the two strains (% entry to closed arm of the elevated plus maze; SD-Veh, 69.76±9.34 vs. 72.30±11.25). WKY rats demonstrated a decrease in both total distance moved in the elevated plus maze and distance moved in the open arms of the elevated plus maze compared to vehicle-treated SD controls (SD-Veh vs. WKY-Veh, p<0.05, **Fig 6.5C&D**). In the open-field test, vehicle-treated WKY rats spent significantly less time in the centre zone of the open-field when compared with SD counterparts indicating anxiogenic-like behaviour (SD-Veh vs. WKY-Veh, p<0.05, **Fig. 6.6A**). WKY rats exhibited decreased total distance moved in the open field (SD-Veh vs. WKY-Veh, **Fig 6.6B&C**).

Intra-peritoneal administration of various doses of URB597 did not alter the duration of time spent in the open arms and % entry into the open arms in SD or WKY rats compared with their respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-URB597, **Fig 6.5A&B**), although a trend towards a decrease in both parameters was seen at 0.2mg/kg dose of URB597 in SD rats. Systemic URB597 administration did not affect locomotor activity of SD or WKY rats on the elevated plus maze (measured as total distance moved and distance moved in the open arms of the elevated plus maze) at any of the doses tested when compared with their respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-URB597, **Fig 6.5 (C&D)**). In addition, intra-peritoneal administration of various doses of URB597 did not affect the duration of time spent in the centre zone of the open field in SD or WKY rats compared with their respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-URB597, **Fig 6.6A**). Similarly, systemic URB597 administration did not alter locomotor activity of SD or WKY rats on the open field (measured as total distance moved in the open field and distance moved in the centre zone of the open field) when compared with their respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-URB597, **Fig 6.6(B&C)**).



**Figure 6.5** Effect of URB597 on anxiety-related behaviours in SD and WKY rats, A) time spent in the open arms of the elevated plus maze (ANOVA: strain:  $F_{(1, 56)}=15.17$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.85$ ,  $p=0.47$  and strain x drug interaction:  $F_{(3, 56)}=0.8$ ,  $p=0.49$ ) and B) % open arm entry (ANOVA: strain:  $F_{(1, 56)}=0.04$ ,  $p=0.88$ ; drugs:  $F_{(3, 56)}=0.63$ ,  $p=0.66$  and strain x drug interaction:  $F_{(3, 56)}=0.34$ ,  $p=0.79$ ) C) total distance moved on the elevated plus maze (ANOVA: strain:  $F_{(1, 56)}=36.81$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.45$ ,  $p=0.72$  and strain x drug interaction:  $F_{(3, 56)}=0.69$ ,  $p=0.56$ ) D) distance moved on the open arm of the elevated plus maze (ANOVA: strain:  $F_{(1, 56)}=16.86$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.66$ ,  $p=0.58$  and strain x drug interaction:  $F_{(3, 56)}=0.69$ ,  $p=0.56$ ); \* $p<0.05$ ; \*\* $p<0.01$  vs. SD-Veh, ^ $p<0.05$  vs. SD-URB-0.1, # $p<0.05$  vs. SD-URB-0.2, ~ $p<0.05$  vs. SD-URB-0.5; Data are mean  $\pm$  SEM ( $n=8$ ); SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle; OA, open arms; EPM, elevated plus maze



**Figure 6.6** Effect of URB597 on anxiety-related behaviours in SD and WKY rats, A) time spent in the centre zone of open field (ANOVA: strain:  $F_{(1, 56)}=7.78$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.56$ ,  $p=0.65$  and strain x drug interaction:  $F_{(3, 56)}=0.38$ ,  $p=0.76$ ) B) total distance moved in the open field (ANOVA: strain:  $F_{(1, 56)}=32.76$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.56$ ,  $p=0.65$  and strain x drug interaction:  $F_{(3, 56)}=0.77$ ,  $p=0.51$ ) C) distance moved in the centre zone of the open field (ANOVA: strain:  $F_{(1, 56)}=8.28$ ,  $p<0.01$ ; drugs:  $F_{(3, 56)}=0.83$ ,  $p=0.48$  and strain x drug interaction:  $F_{(3, 56)}=0.67$ ,  $p=0.57$ ); \* $p<0.05$ ; \*\* $p<0.01$  vs. SD-Veh,  $^p<0.05$  vs. SD-URB-0.1,  $\sim p<0.05$  vs. SD-URB-0.5; Data are mean  $\pm$  SEM ( $n=8$ ); SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle; OF, open field

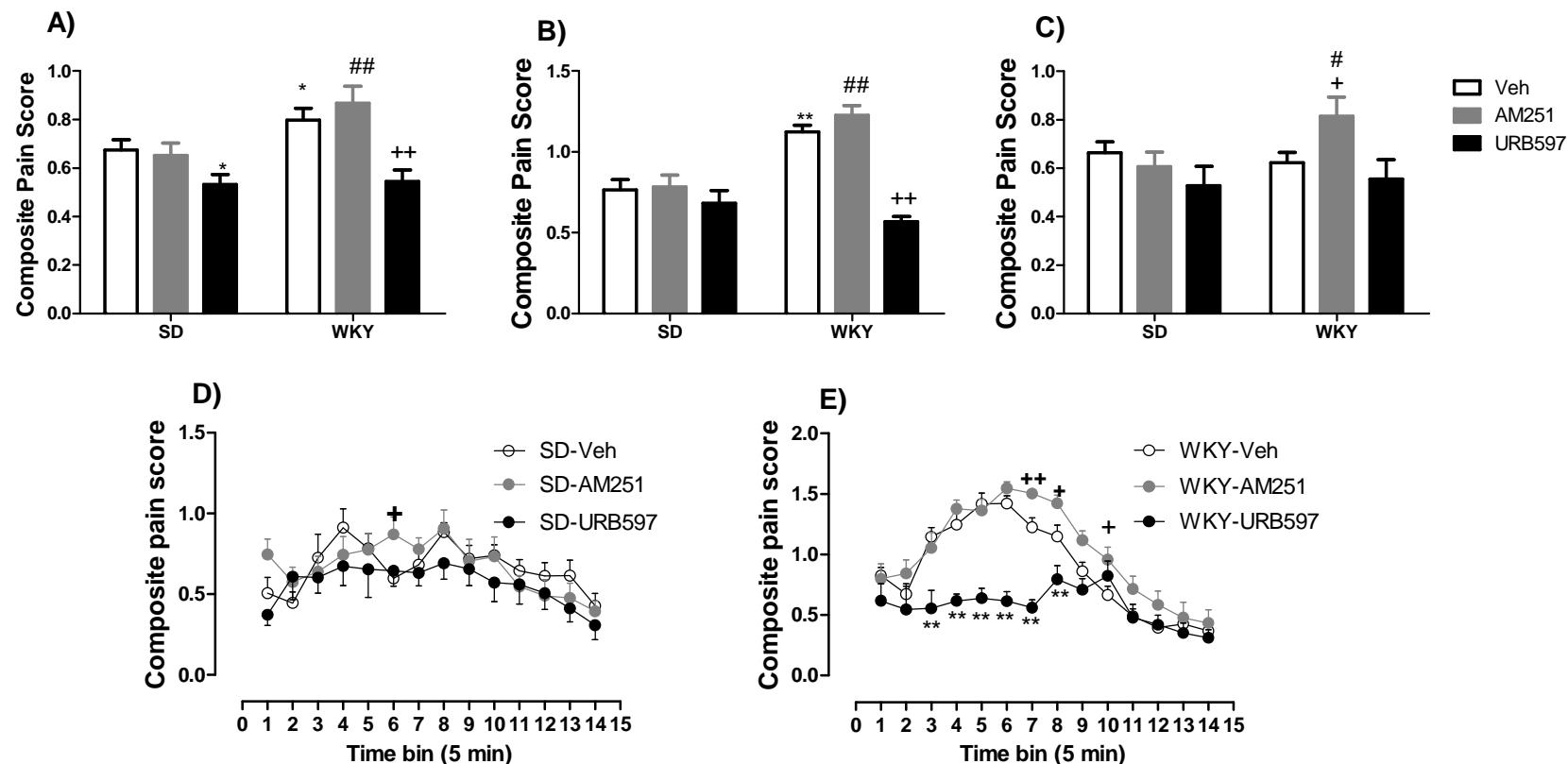
### **6.3.3 Experiment 3**

#### **6.3.3.1 Effect of AM251 or URB597 on nociceptive responding to a persistent inflammatory stimulus in WKY and SD rats**

Intra-plantar formalin administration produced robust licking, biting, shaking and elevation of the right hind paw as indicated by the composite pain score (**Fig 6.7**). Both SD and WKY rats exhibited the classic biphasic response demonstrated by a peak in nociceptive behaviour approximately 10 min following formalin administration, which subsides and was then followed by a second phase of nociceptive behaviour beginning 13-15 min post formalin administration (**Fig 6.7**). Analysis revealed that WKY rats exhibited significantly higher formalin-evoked nociceptive behaviour in both the first and second phase of formalin-induced nociception (SD-Veh vs. WKY-Veh,  $p<0.05$ , **Fig 6.7**). Nociceptive behaviour of both WKY and SD rats was similar beyond 45min post-formalin administration. Furthermore, analysis of area under the curve revealed that WKY rats exhibit enhanced nociceptive responding following intra-plantar formalin when compared to SD controls over the entire 70min (SD-Veh vs. WKY-Veh;  $8.83\pm0.91$  vs.  $11.72\pm1.42$ ,  $p<0.05$ ). Thus, WKY rats display significantly greater formalin-evoked nociceptive behaviour compared with SD counterparts.

Intra-peritoneal administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, AM251, did not affect formalin-induced nociceptive behaviour in SD rats throughout the 70min trial period compared to the vehicle-injected controls (SD-Veh vs. SD-AM251, **Fig 6.7A & C**). However, AM251 significantly enhanced formalin-evoked nociceptive behaviour in WKY rats over the 70min trial period. The majority of AM251-induced enhancement of nociceptive behaviour was seen during the second half of the experiment (i.e. after 35min post-formalin injection) (WKY-Veh vs. WKY-AM251, **Fig 6.7E**). Comparison of AM251-treated SD and WKY rats showed significantly higher nociceptive behaviour in WKY rats compared to SD counterparts (SD-AM251 vs. WKY-AM251, **Fig 6.7A-C**). Systemic administration of the FAAH inhibitor, URB597, significantly reduced formalin-induced nociceptive behaviour in both SD and WKY rats when compared with their respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-URB597,  $p<0.05$ , **Fig 6.7A**). However, the reduction of nociceptive

behaviour achieved by URB597 in WKY rats was larger than that of SD rats (40% reduction in WKY vs. 10% reduction in SD). Thus, there was no difference in nociceptive behaviour between URB597-treated SD and WKY rats (SD-URB597 vs. WKY-URB597, **Fig 6.7A**). The effects of URB597 on nociceptive behaviour in WKY rats were primarily seen during the first 35min of the trial, whereas, in SD rats its effects were sustained throughout the 70min trial (**Fig 6.7 D & E**).



**Figure 6.7** Effects of intra-peritoneal administration of AM251 or URB597 on formalin-evoked nociceptive behaviour in SD and WKY rats A) total over 70 min: (ANOVA: strain:  $F_{(1,57)}=8.37$ ,  $p<0.01$ ; drugs:  $F_{(2,57)}=11.53$ ,  $p<0.01$  and strain x drug interaction:  $F_{(2,58)}=1.93$ ,  $p=0.16$ ) B) 0-35 min post-formalin (ANOVA strain:  $F_{(1,57)}=21.56$ ,  $p<0.01$ ; drugs:  $F_{(2,57)}=22.25$ ,  $p<0.01$  and strain x drug interaction:  $F_{(2,58)}=11.91$ ,  $p<0.01$ ) C) 35-70 min post-formalin (ANOVA strain:  $F_{(1,57)}=1.52$ ,  $p=0.22$ ; drugs:  $F_{(2,57)}=3.32$ ,  $p=6.04$  and strain x drug interaction:  $F_{(2,57)}=2.00$ ,  $p=0.14$ ), \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Veh, + $p<0.05$ , ++ $p<0.01$  vs. WKY-Veh & # $p<0.05$ , ## $p<0.05$  vs. SD-AM251 D&E) 5 min time bin: (repeated measures ANOVA time:  $F_{(13,741)}=31.91$ ,  $p<0.01$ ; time x drug:  $F_{(26,741)}=2.94$ ,  $p<0.01$ , and time x strain interaction:  $F_{(13,741)}=6.69$ ,  $p<0.01$ ; time x drug x strain:  $F_{(26,741)}=2.75$ ,  $p<0.01$ ); \*\* $p<0.01$  WKY-Veh vs. WKY-URB597, + $p<0.05$ , ++ $p<0.01$  SD/WKY-Veh vs. SD/WKY-AM251; Data are mean+SEM (n=10-12); SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle

**6.3.3.2 Effects of systemic administration of AM251 or URB597 on locomotor activity and freezing behaviour in SD and WKY rats on exposure to a novel arena**

During the 10min pre-formalin trial period, WKY and SD rats that received vehicle showed minimal freezing behaviour, although WKY rats exhibited increased duration of freezing when compared with SD rats (SD-Veh vs. WKY-Veh, **Table 6.4**) an effect that did not reach statistical significance. Intraperitoneal administration of 3mg/kg AM251 significantly increased the duration of freezing in both WKY and SD rats compared to respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-AM251, p<0.05, **Table 6.4**). In comparison, URB597 did not affect the duration of freezing in either strain when compared with respective vehicle-treated controls (SD/WKY-Veh vs. SD/WKY-URB597). Vehicle-treated WKY rats displayed significantly lower locomotor activity as measured by the distance moved using Ethovision tracking system or the sum of duration of rearing and grooming in the novel Perspex arena when compared to SD controls (SD-Veh vs. WKY-Veh, p<0.05, **Table 6.4**). AM251 significantly reduced total activity in SD (SD-Veh vs. SD-AM251, p<0.05) rats without affecting total activity in WKY rats or distance moved in both strains. URB597 did not affect either the sum of duration of rearing and grooming or total distance moved in either strain.

Groups	Freezing (S)		Distance moved (cm)		Duration of rearing and grooming (S)	
	Mean	SEM	Mean	SEM	Mean	SEM
SD-Veh	15.86	±5.67	390.34	±33.15	75.65	±9.86
SD-AM251	52.68	±15.81*	360.69	±35.39	48.36	±9.28*
SD-URB597	13.97	±7.34	406.61	±37.77	70.09	±10.43
WKY-Veh	40.79	±7.92	244.46	±34.26*	16.44	±3.78**
WKY-AM251	81.64	±21.23+	206.59	±41.71##	13.99	±3.96##
WKY-URB597	37.33	±11.28	208.89	±45.35~~	17.52	±4.97~~

**Table 6.4** Effect of systemic administration of AM251 or URB597 on general exploratory/locomotor behaviours and freezing in SD and WKY rats during the 10 min pre-formalin trial; Freezing (strain:  $F_{(1, 58)}=6.64$ , p<0.05; drugs:  $F_{(2,58)}=0.06$ , p=6.98 and strain x drug interaction:  $F_{(2,58)}=0.03$ , p=0.97); distance moved (strain:  $F_{(1, 58)}=28.72$ , p<0.01; drugs:  $F_{(2,58)}=0.42$ , p=0.66 and strain x drug interaction:  $F_{(2,58)}=0.27$ , p=0.77) and the sum of duration of rearing and grooming (strain:  $F_{(1, 58)}=60.88$ , p<0.01; drugs:  $F_{(2,58)}=2.18$ , p=0.12 and strain x drug interaction:  $F_{(2,58)}=1.42$ , p=0.25) \*p<0.05 vs. SD-Veh, +p<0.05 vs. WKY-Veh, ##p<0.01 vs. SD-AM251, ~~p<0.01 vs. SD-URB597 (ANOVA followed by Fisher's LSD posthoc test); Data are mean±SEM (n=10-12) SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle

**6.3.3.3 Effect of systemic administration of AM251 or URB597 on locomotor activity, freezing, paw diameter and defecation in the presence of formalin-evoked nociceptive tone in SD and WKY rats**

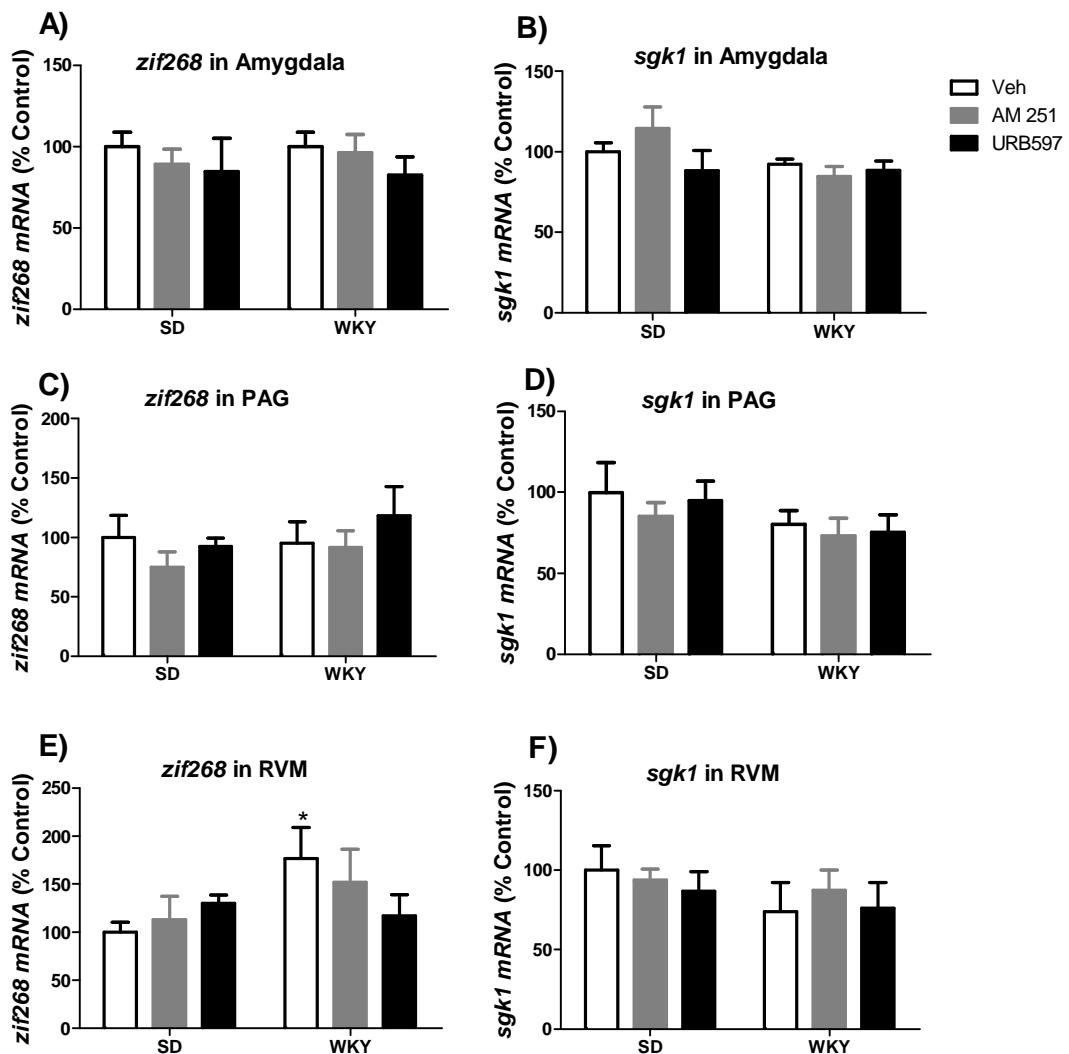
During the 70 min formalin trial, vehicle-treated WKY rats showed a trend for decreased locomotor activity, measured using both automated tracking and manual scoring, although this failed to reach statistical significance (SD-Veh vs. WKY-Veh, **Table 6.5**). Neither AM251 nor URB597 altered the total distance moved of SD and WKY rats compared to vehicle-treated controls. AM251 increased the sum duration of rearing and grooming in formalin-injected SD and WKY rats compared to vehicle-treated rats, although the result in WKY rats failed to reach statistical significance. URB597 administration, in contrast, did not affect the sum duration of rearing and grooming compared to vehicle-treated rats (SD/WKY-Veh vs. SD/WKY-URB). In addition, there was no difference in the total duration of freezing between vehicle-treated SD and WKY rats, and neither URB597 nor AM251 affected the duration of freezing in either strain in the presence of formalin-evoked nociceptive tone (SD-Veh vs. SD-URB/AM251 or WKY-Veh vs. WKY-URB/AM251). Intra-plantar formalin resulted in a similar increase in the diameter of the injected right hind paw in both strains (SD-Veh vs. WKY-Veh), with no effect of drug treatment (SD-Veh vs. WKY-Veh, SD-Veh vs. SD-AM251/URB or WKY-Veh vs. WKY-AM251/URB, **Table 6.5**). Vehicle-treated WKY rats excreted a higher number of faecal pellets compared to respective SD controls (SD-Veh vs. WKY-Veh, p<0.01, **Table 6.5**). However, neither AM251 nor URB597 affected defecation in either strain (SD-Veh vs. SD-AM251/URB or WKY-Veh vs. WKY-AM251/URB).

Groups	Total distance (cm)		Duration of rearing and grooming (S)				Freezing (S)		ΔPaw diameter (mm)		Defecation (number of pellets)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	SD-Veh	15667.52	±2565.73	55.50	±16.10	304.41	±92.92	0.98	±0.06	1.25	±0.38	
SD-AM251	18115.07	±2626.16	109.15	±33.91*	205.78	±96.68	1.35	±0.12	1.40	±0.59		
SD-URB597	11936.99	±1358.43	45.22	±12.20	196.28	±86.43	1.12	±0.08	1.10	±0.42		
WKY-Veh	9812.89	±1768.03	29.69	±4.23	304.86	±116.16	1.18	±0.06	3.17	±0.91**		
WKY-AM251	17290.00	±4789.79	66.00	±11.45	338.76	±100.52	1.14	±0.09	4.40	±1.00		
WKY-URB597	11850.97	±1879.33	36.39	±7.89	419.34	±157.06	1.22	±0.07	2.70	±0.67		

**Table 6.5** Effect of drug treatment (AM251 or URB597) on general exploratory/locomotor behaviours, freezing, defecation and hind paw oedema; Total distance (strain:  $F_{(1, 57)}=1.12$ ,  $p=0.29$ ; drugs:  $F_{(2,57)}=2.73$ ,  $p=0.07$  and strain x drug interaction:  $F_{(2,57)}=0.78$ ,  $p=0.46$ ); the sum of duration of rearing and grooming (strain:  $F_{(1, 57)}=3.54$ ,  $p=0.065$ ; drugs:  $F_{(2,57)}=4.71$ ,  $p=0.013$  and strain x drug interaction:  $F_{(2,57)}=0.48$ ,  $p=0.62$ ); hind paw oedema (strain:  $F_{(1, 58)}=2.12$ ,  $p=0.15$ ; drugs:  $F_{(2,58)}=1.06$ ,  $p=0.36$  and strain x drug interaction:  $F_{(2,58)}=1.64$ ,  $p=0.20$ ) and defecation (strain:  $F_{(1, 58)}=32.0$ ,  $p<0.01$ ; drugs:  $F_{(2,57)}=2.58$ ,  $p=2.26$  and strain x drug interaction:  $F_{(2,58)}=1.17$ ,  $p=0.32$ ); freezing (strain:  $F_{(1, 57)}=1.69$ ,  $p=0.19$ ; drugs:  $F_{(2,57)}=0.06$ ,  $p=0.94$  and strain x drug interaction:  $F_{(2,57)}=0.53$ ,  $p=0.59$ ) \* $p<0.05$ , \*\* $p<0.01$  vs. SD-Veh, (ANOVA followed by Fisher's LSD posthoc test). Data are mean ± SEM (n=12) SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle

**6.3.3.4 Effect of AM251 or URB597 on levels of expression of zif268 and sgk1 mRNA in discrete brain regions in SD and WKY rats**

Vehicle-treated WKY rats demonstrated significantly higher levels of *zif268* mRNA in the RVM compared to SD counterparts (SD-Veh vs. WKY-Veh, p<0.05, **Fig 6.8**), with no such effects observed in the PAG or amygdala. This increase in *zif268* mRNA levels in the RVM of WKY vs. SD rats was not observed in rats treated with either AM251 or URB597. Systemic administration of either AM251 or URB597 had no significant effects on levels of expression of *zif268* mRNA in the amygdala, PAG or RVM, though URB597 administration tended to reduce levels of expression of *zif268* mRNA in the RVM of WKY rats. There was no difference in the levels of *sgk1* mRNA between vehicle-treated SD and WKY rats in the amygdala, PAG and RVM. The expression of this gene in any of the brain regions was unaffected by systemic administration of AM251 or URB597 in either SD or WKY rats.



**Figure 6.8** Effects of AM251 or URB597 on levels of expression of *zif268* and *sgk1* mRNA in discrete brain regions of SD and WKY rats A) *zif268* in amygdala (strain:  $F_{(1, 36)}=0.25$ ,  $p=0.62$ ; drugs:  $F_{(2,36)}=1.51$ ,  $p=0.24$  and strain x drug interaction:  $F_{(2,36)}=0.13$ ,  $p=0.88$ ) B) *sgk1* in amygdala (strain:  $F_{(1, 36)}=3.08$ ,  $p=0.08$ ; drugs:  $F_{(2,36)}=0.87$ ,  $p=0.43$  and strain x drug interaction:  $F_{(2,36)}=1.64$ ,  $p=0.21$ ) C) *zif268* in PAG (strain:  $F_{(1, 36)}=0.82$ ,  $p=0.37$ ; drugs:  $F_{(2,36)}=0.87$ ,  $p=0.43$  and strain x drug interaction:  $F_{(2,36)}=0.43$ ,  $p=0.65$ ) D) *sgk1* in PAG (strain:  $F_{(1, 36)}=3.08$ ,  $p=0.08$ ; drugs:  $F_{(2,36)}=0.43$ ,  $p=0.66$  and strain x drug interaction:  $F_{(2,36)}=0.062$ ,  $p=0.94$ ) E) *zif268* in RVM (strain:  $F_{(1, 36)}=4.2$ ,  $p=0.05$ ; drugs:  $F_{(2,36)}=0.04$ ,  $p=0.96$  and strain x drug interaction:  $F_{(2,36)}=1.05$ ,  $p=0.36$ ) F) *sgk1* in RVM (strain:  $F_{(1, 36)}=0.22$ ,  $p=0.80$ ; drugs:  $F_{(2,36)}=1.58$ ,  $p=0.22$  and strain x drug interaction:  $F_{(2,36)}=0.27$ ,  $p=0.76$ ); \* $p<0.05$  vs. SD-Veh (ANOVA followed by Fisher's LSD posthoc test); Data are mean $\pm$ SEM (n=7) SD, Sprague-Dawley; WKY, Wistar-Kyoto; Veh, Vehicle

#### **6.4 Discussion**

The present study demonstrated that WKY rats exhibited enhanced nociceptive responding to both thermal and inflammatory noxious stimuli and exhibited enhanced anxiety-and fear-related behaviour compared to SD counterparts, thus confirming ARH in this strain of rat. Increasing doses of the CB<sub>1</sub> receptor antagonist/inverse agonist, AM251, or the FAAH inhibitor URB597, enhanced the response latency to the thermal stimulus in WKY rats without affecting responses in SD rats, thereby attenuating ARH to a thermal stimulus. AM251 (3mg/kg) enhanced and reduced anxiety-related behaviours in SD and WKY rats respectively; while in comparison URB597 did not alter anxiety-related behaviours in either strain. Although AM251 did not affect formalin-evoked nociceptive behaviour in SD rats, it enhanced formalin-evoked nociception in WKY rats. In contrast, URB597 reduced formalin-evoked nociceptive behaviour in both SD and WKY rats, and attenuated ARH in WKY rats. ARH in WKY rats was associated with increased expression of *zif268* mRNA in the RVM compared to SD rats, an effect not altered by AM251 or URB597.

The present study demonstrated that systemic administration of AM251 at 3mg/kg resulted in anxiety-like behaviour in the SD rats; whereas, it was anxiolytic in WKY rats. Anxiogenic effects of AM251 have been shown in the OF or EPM in mice (Haller *et al.*, 2004; Patel *et al.*, 2006a; Rodgers *et al.*, 2005) and SD rats (Sink *et al.*, 2010) at similar doses to that used in the current study. However, it should also be noted that other studies have shown no effect of AM251 on anxiety-related behaviour (Micale *et al.*, 2008; Thiemann *et al.*, 2009) in mice. Direct comparison with these studies is not possible as most previous studies were conducted on mice. To our knowledge, no other study investigated effect of AM251 on anxiety-related behaviour in WKY rats. The differential effect of CB<sub>1</sub> receptor antagonism in SD and WKY rats suggests that the endocannabinoid system may underlie the altered stress-induced responding in the two strains. It is possible that endocannabinoids in SD rats may elicit a tonic effect of maintaining an anxiety free (or low anxiety) state; whereas, in WKY rats, endocannabinoids may mediate the anxiety-related behaviour seen in these animals. In the previous chapter, it was shown that levels of endocannabinoids

at baseline in the vl and IPAG are higher in WKY when compared to SD rats. Cannabinoids induce diverse responses on anxiety- and fear-related behaviours. Generally, low doses tend to induce anxiolytic-like effects, whereas high doses often cause the opposite (Moreira *et al.*, 2010). Thus, it is possible that increased endocannabinoid levels in WKY rats might be mediating the anxiogenic-like behaviour through activation of CB<sub>1</sub> receptors and that blocking CB<sub>1</sub> receptors attenuated this behaviour. However, it should be noted that the FAAH inhibitor, URB597, did not enhance anxiety-related behaviour in WKY or SD rats. This is in contrast with previous reports where systemic URB597 resulted in anxiolytic-like behaviour (Kathuria *et al.*, 2003; Moise *et al.*, 2008; Moreira *et al.*, 2008; Naidu *et al.*, 2007; Patel *et al.*, 2006a; Scherma *et al.*, 2008). The discrepancy between these findings could be attributed to the different experimental conditions used including strains, model employed and time points of drug administrations. In addition, it has been reported that systemic administration of URB597, unlike benzodiazepines, does not produce robust anxiolytic-like effects when the aversiveness of testing procedures is minimised by altering parameters such as handling, habituation or use of illumination during testing (Haller *et al.*, 2009).

Similar to previous studies (Burke *et al.*, 2010) and as shown in Chapter 5, WKY rats demonstrated hyperalgesia to a noxious thermal stimulus. This ARH was attenuated dose dependently by systemic administration of the FAAH inhibitor, URB597. This is in line with previous reports showing analgesic effect of URB597 to thermal (Hasanein, 2009; Hasanein *et al.*, 2008; Kathuria *et al.*, 2003) stimulus in Wistar and SD rats. Intriguingly, administration of the CB<sub>1</sub> receptor antagonist/inverse agonist, AM251, also dose dependently attenuated thermal hyperalgesia in WKY rats. Previously, analgesic effects of rimonabant, a CB<sub>1</sub> receptor antagonist/inverse agonist and analogue of AM251 were demonstrated when administered systemically (Beaulieu *et al.*, 2000; Costa *et al.*, 2005) or into the BLA (Hasanein *et al.*, 2007; Roche *et al.*, 2010; Roche *et al.*, 2007). In contrast, direct administration of AM251 into the BLA did not have an effect on formalin-evoked nociception (Hasanein *et al.*, 2007; Roche *et al.*, 2010). It is possible that these effects of systemic AM251 on thermal hyperalgesia may result from an action of the drug at alternative targets to the

CB<sub>1</sub> receptor. For example, *in vitro* evidence suggests that AM251 can act as an agonist for GPR55 (Henstridge *et al.*, 2009; Kapur *et al.*, 2009; Ryberg *et al.*, 2007). However, a recent study showed that GPR55 knockout mice failed to demonstrate hyperalgesia associated with inflammatory and neuropathic pain suggesting a role for GPR55 in mediating hyperalgesia rather than analgesia (Staton *et al.*, 2008). AM251 was also shown to activate TRPA1 receptors though this was associated with anti-analgesic effect (Patil *et al.*, 2011). In the present experiment, the analgesic effect of AM251 was only limited to the hot plate test as AM251 induced hyperalgesia rather than analgesia in the formalin test. Previous studies have demonstrated that, hot-plate latencies, but not formalin test scores or tail-flick latencies, were significantly increased in rats with medial frontal cortex lesions suggesting a differential supraspinal processing for different forms of noxious stimuli (Pastoriza *et al.*, 1996). The lack of antinociceptive effect of URB597 in SD rats is not consistent with previous work demonstrating antinociceptive effect of this drug in the tail flick test in SD rats (Hassanien *et al.*, 2008) and Wistar rats (Hasanein *et al.*, 2009) as well as in the hot plate test in Wistar rats (Kathiuria *et al.*, 2003). However, differences in strain and pain model employed could explain these disparities. The lack of effect on nociceptive behaviour of AM251 in SD rats suggests that the endocannabinoid system might not have tonic effects in this strain. Even though previous work showed that AM251 can prevent antinociceptive effects on the tail flick test of endocannabinoid agonists (Hasanein *et al.*, 2008; Hasanein *et al.*, 2009), studies investigating the effect of AM251 *per se* on thermal nociception are lacking. The differential response of SD vs. WKY rats to AM251 and URB597 on thermal nociception suggests differences in endocannabinoid system function between the two strains and extends the findings presented in Chapter 5.

The ARH to formalin evoked nociception seen in WKY rats is in agreement with our finding in Chapter 5 and past work showing this phenomenon (Burke *et al.*, 2010). Systemic administration of AM251 enhanced ARH to formalin-evoked nociception in WKY rats without affecting formalin-evoked nociception in SD rats. Previous studies showed that AM251 had no effect (Hasanein, 2009; Jayamanne *et al.*, 2006; Naidu *et al.*, 2010) or was slightly hyperalgesic (Maione

(*et al.*, 2007) on formalin-evoked nociception. URB597, in contrast, attenuated formalin-evoked nociception in both strains though its effects were more pronounced in WKY rats. This is consistent with previous work demonstrating an analgesic effect of URB597 on formalin-evoked nociception (Hasanein, 2009). In addition, other FAAH inhibitors such as MAFP (Ates *et al.*, 2003), flurbiprofen (Ates *et al.*, 2003), propofol (Guindon *et al.*, 2007b), AA-5HT (Maione *et al.*, 2007), OMDM106 (Ortar *et al.*, 2007) have been shown to be antinociceptive in the formalin test. The AM251-induced enhancement and URB597-induced attenuation of hyperalgesia in WKY rats together suggest that enhanced activity of endocannabinoids acting through CB<sub>1</sub> receptors might be essential to prevent ARH. This is in line with previous reports showing that pharmacological stimulation of CB<sub>1</sub> receptors using ACEA (Shen *et al.*, 2010) or WIN 55,212-2 (Hong *et al.*, 2009) reduced stress-induced visceral hyperalgesia; whereas, antagonism of these receptors with rimonabant (Shen *et al.*, 2010) elicited the opposite effect, suggesting a role for the endocannabinoid system in stress-induced visceral hyperalgesia. To our knowledge, our findings are the first demonstration of a role for the endocannabinoid system in ARH to somatic noxious stimuli. The fact that systemic administration of URB597 was antinociceptive in both SD and WKY rats suggests that this is an effect of the drug on nociception *per se* rather than a specific effect on ARH. However, the effect of URB597 in WKY rats was so profound that the difference in the response to formalin-induced nociception between the two strains was completely abolished. This suggests that diminished concentrations of, or activity of, endocannabinoids and related lipids might contribute to the behavioural expression of ARH. In the previous chapter (chapter 5), we demonstrated that following intra-plantar formalin injection, endocannabinoid levels were higher in most parts of the PAG of WKY rats but lower in the RVM when compared to SD rats. It is possible that the elevated endocannabinoid levels in the PAG function as part of an endogenous compensatory mechanism to counteract the hyperalgesia since URB597, which is known to enhance levels of endocannabinoids and related lipids, attenuated the hyperalgesia. In addition, the lower levels of endocannabinoids and related lipids in the RVM might indicate deficient or blunted responsivity of the endocannabinoid system in the RVM in WKY rats and URB597 might be offsetting this effect to attenuate the

hyperalgesia. Moreover, at the dose employed, URB597 had no effect on general exploratory/ locomotor behaviour in either strain and the decrease in total activity following administration of AM251 in SD rats in the 10min pre-formalin trial period cannot explain the effect of AM251 observed on ARH, indicating a specific effect of both drugs on hyperalgesia.

It is possible that some of the effects of URB597 observed in the present study may arise as a consequence of FAAH substrate activity at non-CB<sub>1</sub> receptors. Evidence exists suggesting an important role of AEA activity at the transient receptor potential vanilloid 1 channel (TRPV1) in the regulation of both pain (Palazzo et al., 2008; Maione et al., 2006) and aversion (Moreira et al., 2009; Terzian et al., 2009). Indeed, stimulation of TRPV1 receptors in the PAG by AEA was shown to induce antinociception (Maione et al., 2006). Though Suplita et al. (2005) showed that TRPV1 was not involved in unconditioned SIA, another form of anxiety-related modulation of pain in rats, a role for the TRPV1 receptor in stress-induced visceral hyperalgesia has been described (Hong et al., 2009). Water avoidance stress was accompanied by enhanced expression of TRPV1 receptors in DRG neurons and the TRPV1 receptor antagonist, capsazepine, prevented water avoidance stress-induced visceral hyperalgesia (Hong et al., 2009). In addition, the upregulation of TRPV1 receptor levels in water avoidance stress rats was attenuated by chronic treatment with the selective CB<sub>1</sub> receptor agonist WIN 55,212-2, which at the same time prevented stress-induced visceral hyperalgesia, supporting a potential interaction between CB<sub>1</sub> and TRPV1 receptors in stress-induced hyperalgesia. Collectively, these results indicate that TRPV1 receptors might be facilitating stress-induced hyperalgesia and that CB<sub>1</sub> receptors might be protective against stress-induced hyperalgesia. Future work is required to determine the role of TRPV1 receptors in ARH.

The expression of *zif268* was increased in the RVM in association with ARH, indicating an important role of this brain region in ARH and suggesting that *zif268* in the RVM may be a possible molecular correlate of this phenomenon. These data are consistent with past reports showing the importance of the RVM

in stress-induced hyperalgesia to mechanical (Reynolds *et al.*, 2011), inflammatory (Imbe *et al.*, 2010) and thermal hyperalgesia (Imbe *et al.*, 2004; Senba *et al.*, 2008). Using simultaneous single-cell recording and functional analysis, Martenson *et al.* (2009) showed that stimulation of the dorsomedial nucleus of the hypothalamus, a critical component of the stress response, triggers thermal hyperalgesia by recruiting pain-facilitating neurons in the RVM ("ON-cells") demonstrating a top-down activation of brainstem pain-facilitating neurons as a possible neural circuit for stress-induced hyperalgesia (Martenson *et al.*, 2009). The data presented here do not provide any conclusive evidence that the increased RVM *zif268* expression associated with ARH is under the control of the endocannabinoid system as neither blockade of the CB<sub>1</sub> receptor nor inhibition of FAAH, significantly altered its expression. However, it is worth noting that the increased *zif268* expression observed in vehicle-treated WKY rats vs. SD controls was not seen in WKY rats receiving either AM251 or URB597. One interpretation of these data therefore is that these pharmacological manipulations of the endocannabinoid system prevented the increased in RVM *zif268* levels that accompany ARH. Though the PAG (Devall *et al.*, 2010; Rosenberger *et al.*, 2009) and amygdala (Ait-belgnaoui *et al.*, 2009; Chung *et al.*, 2009) are implicated in stress-induced hyperalgesia, expression of *zif268* was not affected in these brain regions in association with ARH in the present experimental conditions. It is possible that changes in *zif268* expression might occur at a different time point or following different form of noxious stimuli. It is also possible that other molecular correlates such as *c-fos* (Ait-belgnaoui *et al.*, 2009; Devall *et al.*, 2010) may be responsible for mediating ARH in these brain regions.

In conclusion, the present study demonstrated that blockade of CB<sub>1</sub> receptors affected anxiety-related behaviour in a differential manner in SD and WKY rats suggesting a differential response of the endocannabinoid system in the two strains in response to aversion. In addition, both blockade of CB<sub>1</sub> receptor and FAAH resulted in a differential response to thermal and inflammatory nociception in SD and WKY rats. The data suggest a possible protective role of the endocannabinoid system against ARH. Moreover, the results implicate *zif268* in the RVM as a possible molecular correlate of ARH. These data further

***Chapter 6: Effects of systemic inhibition of CB<sub>1</sub> receptor or FAAH on anxiety-related hyperalgesia and expression of plasticity-related genes zif268 and sgk1***

advance our understanding of underlying neurochemical and molecular mechanisms mediating pain, anxiety and ARH.

**Chapter 7: General discussion**

Chronic pain is a major clinical problem affecting the quality of life of a large number of people world-wide and costing the world economy immensely. Better understanding of mechanisms of pain is key to advance existing treatment strategies for chronic pain. It is now known that different forms of aversion affect pain processing in a differential manner. Understanding the neurochemical and molecular mechanisms of such endogenous modulation of pain has both physiological and therapeutic significance. The work presented in this dissertation addressed the particular role of the endocannabinoid system in emotional modulation of pain. While Chapters 2-4 focussed on identifying neural substrates and molecular correlates of FCA, Chapters 5 and 6 investigated the role of the endocannabinoid system in ARH.

There are 4 major contributions of the work presented in this thesis to our understanding of the interactions between pain and anxiety/fear: 1) Generation of a comprehensive profile of the alterations in levels of endocannabinoids and NAEs that are associated with behavioural expression of FCA, conditioned fear and pain. 2) Demonstration of an important role of the endocannabinoid system in the dIPAG in the mediation of FCA. 3) Investigation of the extent to which Erk phosphorylation and levels of *zif268* mRNA and *sgk1* mRNA are associated with the expression of FCA, pain-and fear-related behaviours with and without endocannabinoid modulation. 4) Characterization of behavior and endocannabinoid system expression and function in a genetic model of ARH (the WKY rat). Together, these studies advance our understanding of the neurobiological mechanisms underpinning the bidirectional effects of stress, fear and anxiety on pain.

The work presented in Chapter 2 demonstrated a differential response of endocannabinoids and related lipids during exposure to a conditioned stressor, noxious inflammatory stimulus or during expression of FCA, in discrete brain regions including the PAG, BLA, hippocampus, insular cortex, PFC and RVM in rats. These data provide a foundation upon which to design further mechanistic studies aimed at elucidating the neural substrates and neurochemical mechanisms underpinning endocannabinoid-mediated FCA.

## **References**

Tissue concentrations of the endocannabinoids, AEA and 2-AG, and the related ‘entourage’ NAEs, PEA and OEA, were primarily increased in the PAG, BLA and RVM of rats sacrificed 3min following re-exposure to a context previously paired with footshock, suggesting that conditioned fear engages/mobilises endocannabinoids and NAEs (see **Table 7.1**). These findings corroborate previous work which has shown footshock stress induced increases in levels of 2-AG and AEA in the rat dlPAG (Hohmann *et al.*, 2005) and conditioned stress induced increases in AEA and 2-AG levels in the mouse BLA (Marsicano *et al.*, 2002). Interestingly, our data represent the first report of the effects of conditioned fear on levels of OEA and PEA in the brain (in the dlPAG and BLA) and highlight the need to further investigate the role of these compounds in fear/anxiety-related behaviour. Given the very significant overlap in the neural substrates, brain regions and circuitry involved in pain and fear/anxiety, it is possible that fear-induced alterations in neuronal activity, neurotransmission and/or neurochemistry within these regions influence pain-related behaviour. We have shown that expression of FCA was associated with increased levels of AEA in the left IPAG and right dlPAG. Previously, Hohmann *et al.*, reported that increased endocannabinoid levels in the dlPAG, following exposure to stress, mediate SIA through activation of the descending inhibitory pain pathway that projects to the DHSC (Hohmann *et al.*, 2005; Suplita *et al.*, 2005; Suplita *et al.*, 2006). Fear-related increases in endocannabinoids in regions such as the dlPAG may play a key role in mediating or modulating conditioned fear behaviours as well as FCA. Thus, we hypothesised that the increased AEA observed in the dlPAG mediates the suppression of pain by conditioned psychological stress. Indeed, the present study demonstrates for the first time that direct administration of rimonabant, a CB<sub>1</sub> receptor antagonist/inverse agonist, into the right dlPAG, prevents FCA. In addition, intra-dlPAG administration of the FAAH inhibitor, URB597, showed a strong trend to enhance FCA. Finn and colleagues provided the first evidence for a role of CB<sub>1</sub> receptors in mediating FCA (Finn *et al.*, 2004) and subsequent work also revealed enhanced cannabinoid-mediated FCA following systemic administration of URB597 (Butler *et al.*, 2008, 2011a). Unlike SIA, where a role for the endocannabinoid system in the PAG (Hohmann *et al.*, 2005; Suplita *et al.*, 2005), BLA (Connell *et al.*, 2006), RVM (Suplita *et al.*, 2005) and spinal cord

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(Suplita *et al.*, 2006) has been demonstrated, until recently no particular brain region has been implicated in endocannabinoid mediated-FCA. In fact, work has shown that CB<sub>1</sub> receptors in the BLA may not be responsible for endocannabinoid-mediated FCA (Roche *et al.*, 2009; Roche *et al.*, 2007). The results of the work presented in Chapter 2 suggest that elevations of AEA in the dlPAG may be responsible for mediating expression of FCA, possibly through activation of CB<sub>1</sub> receptors, which in turn may lead to subsequent disinhibition of output neurons and activation of the descending inhibitory pain pathway (de Novellis *et al.*, 2005; Vaughan *et al.*, 2000). However, caution should be exercised when interpreting the effects of AEA as it is also possible that alternative targets such as TRPV1 (Maione *et al.*, 2006; Moreira *et al.*, 2009a; Palazzo *et al.*, 2008; Terzian *et al.*, 2009) could mediate the effects of AEA in the dlPAG on FCA. Future experiments, involving intra-dlPAG blockade of TRPV1, would be helpful to rule out the role of this receptor in FCA.

**Table 7.1** Summary of alterations in levels of endocannabinoids and related lipids during nociception, conditioned fear and their combination

		FCA	Fear	Nociception	Nociception during fear
Side	Brain region	FC-Form vs NFC-Form	FC-Sal vs NFC-Sal	NFC-Form vs NFC-Sal	FC-Form vs FC-Sal
left	dlPAG	↓ 2-AG	↑ PEA		↓↓ 2-AG,OEA
right	dlPAG	↑ AEA	↑ all		
left	vIPAG		↑ 2-AG		↓ 2-AG
right	vIPAG		↑ 2-AG		↓ 2-AG
left	IPAG	↑ AEA	↓ AEA	↓ AEA	
right	IPAG			↓ 2-AG	
left	BLA		↑ all but 2-AG		
right	BLA		↑ OEA		
	RVM		↑↑ AEA, 2-AG		↓ 2-AG
left	vlHipp				
right	vlHipp				
left	dlHipp		↑ PEA		
right	dlHipp				
left	Ins			↑ AEA	
right	Ins				
	PFC			↑↑ 2-AG,PEA	

FC, fear-conditioned; NFC, non fear-conditioned; Sal, Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

## **References**

Supporting and extending previous reports showing the antinociceptive effects of URB597 administered systemically (Hasanein, 2009; Jayamanne *et al.*, 2006; Kinsey *et al.*, 2009; Naidu *et al.*, 2010; Russo *et al.*, 2007) and intra-vIPAG (Maione *et al.*, 2006), the work presented herein showed that intra-dlPAG administration of URB597 was antinociceptive in the rat formalin model of tonic persistent pain. This finding suggests that a FAAH substrate (e.g. AEA, OEA or PEA) in the dlPAG modulates nociceptive responding, probably by modulating the descending inhibitory pain pathway at this level. These results could have direct therapeutic implications, though the invasiveness of the mode of administration (i.e. intra dlPAG) would of course limit the extent to which such an approach could be translated to humans. Intra-dlPAG administration of URB597 did not have any significant effect on contextually-induced conditioned fear behaviours, even though it tended to decrease fear behaviour with increasing concentrations. This is contrary to previous work demonstrating anxiolytic effects of URB597 (Hill *et al.*, 2007; Kathuria *et al.*, 2003; Lisboa *et al.*, 2008; Moise *et al.*, 2008; Moreira *et al.*, 2008; Naderi *et al.*, 2008; Naidu *et al.*, 2007; Patel *et al.*, 2006b; Scherma *et al.*, 2008). However, differential modulation of anxiety depending on the dose of URB597 (Rubino *et al.*, 2008b; Scherma *et al.*, 2008) or aversiveness of testing procedures (Haller *et al.*, 2009) has been shown. As discussed above, effects of URB597 could as well be due to effects of AEA on alternate targets and therefore may not be solely attributed to effects at the CB<sub>1</sub> receptor. The use of FAAH inhibitors such as URB597 for the treatment of pain/anxiety is appealing as this compound is devoid of CNS side effects, such as the psychomimetic side effects and abuse potential that are associated with potent CB<sub>1</sub> receptor agonists (Justinova *et al.*, 2008). While there are not any clinical trials using URB597, a recent study investigated PF-04457845, another FAAH inhibitor, for its efficacy in treating pain in patients with osteoarthritis of the knee, results of which have not as yet been published ([clinicaltrial.gov](http://clinicaltrial.gov)).

In line with previous reports that amygdalar MAPK activation is important in the acquisition and consolidation of conditioned fear (Di Benedetto *et al.*, 2008; Duvarci *et al.*, 2005; Schafe *et al.*, 2000), the work presented herein has shown that fear conditioning significantly increases expression of pErk1 in the right

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BLA of rats. The fear-induced increase in pErk1 was accompanied by increased levels of OEA, and a strong trend for increased AEA, in the right BLA. Given that the expression of conditioned fear is associated with increased pErk expression in the BLA, in a CB<sub>1</sub> receptor-dependent manner (Cannich *et al.*, 2004), increased levels of AEA and OEA (via the ‘entourage’ effect) during conditioned-fear may activate CB<sub>1</sub> receptors to result in increased levels of pErk1 in the BLA. In the present experiment, FCA, which was associated with increased levels of AEA in the right dlPAG, was also accompanied by a strong trend towards increased levels of pErk1/2 in the right dlPAG at both 3min and 15min following re-exposure to the conditioned arena. These results suggest the possibility that AEA-induced activation of Erk in the dlPAG following fear conditioning might mediate FCA. However, neither the reversal nor the enhancement of FCA by intra-dlPAG rimonabant and URB597 respectively, were accompanied by alterations in the expression of relative pErk1/2 in the dlPAG. To determine if increased expression of pErk in the dlPAG is causally related to FCA, future work involving intra-dlPAG administration of an inhibitor of MAPK/Erk (e.g. U0126) would be informative. In agreement with previous work showing an increase in pErk1/2 in the amygdala (Butler *et al.*, 2008), FCA was associated with increased pErk1 in the right BLA, suggesting a possible role for pErk1 in the BLA in the mediation of FCA. However, Butler and colleagues showed that both rimonabant-mediated attenuation and URB597-mediated enhancement of FCA were associated with reduced pErk2 in the amygdala, questioning the role of pErk1/2 in this brain region in FCA. In addition, bilateral BLA administration of U0126, inhibitor of MAPK/Erk signalling, reduced the formalin-evoked nociceptive behaviour in non fear-conditioned rats but did not affect FCA (Butler *et al.*, 2009). In addition to influencing gene transcription, MAPK signalling also modulates cellular activity at the non-transcriptional level, including inhibition of voltage-gated Ca<sup>2+</sup> (Fitzgerald, 2000) and K<sup>+</sup> (Hu *et al.*, 2003) channels. Thus, MAPK signalling in the PAG and BLA could inhibit Ca<sup>2+</sup> or K<sup>+</sup> currents in this region and consequently alter the activity of the descending amygdala-PAG-RVM pathway.

## **References**

The present studies also investigated novel molecular correlates of endocannabinoid-mediated FCA. FCA was accompanied by an attenuation of the formalin-evoked increase in the expression of *zif268* in the ipsilateral DHSC (and a strong trend in a similar direction for *sgk1*) suggesting that fear-induced activation of the descending inhibitory pain pathway may result in suppression of pain-evoked *zif268* expression within neurons of the DHSC. This is strong evidence that FCA is manifested not only at the behavioural level but is likely to be due to changes in the activity of neurones involved in nociceptive processing. The present experiments also provided evidence that molecular changes that accompany FCA appear to be due to the conditioned fear *per se* rather than the footshock exposure the previous day as there were no associated alterations in expression of either *zif268* or *sgk1* in any of the regions investigated in FCD rats (i.e. rats that received footshock but were re-exposed to a different context). Pharmacological blockade of the CB<sub>1</sub> receptor by systemic administration of AM251 attenuated FCA and prevented the fear-induced reduction of *zif268* expression in the ipsilateral DHSC in formalin-treated rats. These findings indicates that *zif268* expression in the DHSC could be an important molecular mediator of this CB<sub>1</sub>-dependent and endocannabinoid-mediated FCA. Our findings here support a previous report which showed that the DHSC is important for endocannabinoid-mediated suppression of pain responding following exposure to unconditioned stress (Suplita *et al.*, 2006) and extend this finding by suggesting that the DHSC is also a relevant structure in endocannabinoid-mediated suppression of pain responding induced by psychological, conditioned stress/fear. Future work using techniques such as immunohistochemistry or *in situ* hybridization to localize which neurons *zif268* expression is altered is warranted.

*zif268* expression has been shown to be increased in the neurons of the hippocampus, anterior cingulate cortex, thalamus and PAG following noxious stimuli (Pagano *et al.*, 2011; Wei *et al.*, 1999; Wei *et al.*, 2000) and to date increased *sgk1* expression following noxious stimuli has only been shown in the DHSC (Geranton *et al.*, 2007). Although our studies suggest a lack of effect of intraplantar formalin injection on *zif268* or *sgk1* expression in supraspinal areas such as the amygdala, PAG or RVM, it is possible that, alterations in these

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genes may occur at a time point other than those examined here or following a different form of noxious stimulus. The increased expression of *zif268* in the RVM, and a strong similar trend in the amygdala, during conditioned-fear support and extend previous work (Hall *et al.*, 2001; Lonergan *et al.*, 2010; Busti *et al.*, 2011; Hall *et al.*, 2000; Hall *et al.*, 2001; Malkani *et al.*, 2000; Perez-Villalba *et al.*, 2008; Ressler *et al.*, 2002; Rosen *et al.*, 1998a) and suggest *zif268* as an important molecular correlate of conditioned fear in these brain regions.

The last two experiments of this thesis examined the role of the endocannabinoid system in ARH using two rat strains with different baseline emotionality. These experiments demonstrated that WKY rats exhibited enhanced anxiety-related behaviour and showed enhanced nociceptive responding to acute and persistent noxious stimuli compared to SD rats, supporting the contention that WKY rats may represent a genetic model of ARH which can be used to understand neurochemical and molecular mechanisms of anxiety-pain co-morbidity. In addition, the two rat strains differed with respect to baseline levels of endocannabinoids, NAEs and expression of *CB<sub>1</sub>*, *FAAH* and *MAGL mRNA* in discrete brain regions involved in modulating emotional and pain processes. We have shown here that the anxiety-related phenotype in WKY rats was accompanied by an increase in some analytes in the vIPAG, IPAG and RVM (see **Table 7.2**). The differences in baseline levels and/or the differential response of endocannabinoids and NAEs in supraspinal structures that modulate nociceptive responding in WKY and SD rats in response to noxious stimuli might be responsible for the difference in baseline emotionality and hypersensitivity to noxious stimuli between the two strains. The increased levels of endocannabinoids/NAEs in discrete brain regions of WKY rats may be a compensatory physiological mechanism to counteract the enhanced anxiety- and pain-related behaviour that is observed in the WKY rats. Alternatively elevated endocannabinoid levels may induce anxiety-related behaviour in WKY rats that could be mediated by either CB<sub>1</sub> receptors (Moreira *et al.*, 2010) or TRPV1 receptors (Campos *et al.*, 2009; Terzian *et al.*, 2009; Rubino *et al.*, 2008b; Santos *et al.*, 2008). Our finding that AM251-induced enhancement of formalin-evoked nociceptive behaviour and URB597-induced attenuation of the

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hyperalgesic response to formalin in WKY rats together suggest that decreased activity of endocannabinoids acting through CB<sub>1</sub> receptors might be responsible for ARH, providing the first demonstration of a role for the endocannabinoid system in ARH to a somatic noxious stimulus. Such a demonstration could have direct therapeutic implications as inhibiting FAAH and/or MAGL could represent viable future therapeutic strategies for chronic pain disorders co-existing with anxiety or exacerbated/precipitated by stress. It is possible that some of the effects of URB597 observed in the present study may arise as a consequence of FAAH substrate activity at non-CB<sub>1</sub> receptors such as TRPV1, PPAR or GPR55. As a role for peripheral TRPV1 receptors in stress-induced visceral hyperalgesia has been described (Hong *et al.*, 2009), future work targeting central TRPV1 is required to determine the potential role of these receptors in ARH and the interaction with CB<sub>1</sub> receptors in this response.

Previously, both upregulation (Reich *et al.*, 2009; Robinson *et al.*, 2010; Shen *et al.*, 2010) and downregulation (Hong *et al.*, 2009; Hill *et al.*, 2005; Reich *et al.*, 2009) of CB<sub>1</sub> receptors have been reported following stress. While the former could be part of a compensatory response to counteract stress-induced hypersensitivity, the latter could be causally related to hypersensitivity. In the present study, CB<sub>1</sub> mRNA expression was higher in the dlPAG, BLA and RVM but lower in the DHSC of WKY rats. Whether the changes in CB<sub>1</sub> receptor expression observed in the spinal and suraspinal neural structures of WKY rats have a protective or facilitatory role on ARH will require further study. Work involving intracranial administration of pharmacological agents which target CB<sub>1</sub>, MAGL or FAAH could be revealing in this respect. The present work provides no direct evidence for the role of the endocannabinoid system in specific brain regions in mediating ARH. However, a role for the RVM in ARH has been demonstrated, as shown by decreased endocannabinoids and increased expression of *zif268* in association with ARH. This is in keeping with past reports showing the importance of the RVM in stress-induced hyperalgesia (Imbe *et al.*, 2004; Imbe *et al.*, 2010; Reynolds *et al.*, 2011; Senba *et al.*, 2008). Future work is needed to elucidate neural substrates important in the role of the endocannabinoid system in ARH. The increased expression of *zif268* in association with ARH also suggests that *zif268* in the RVM could be an

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important molecular correlate of ARH. However, whether or not *zif268* has causal role in ARH should be addressed in future studies. For example, investigating the effect of *zif268* inhibition in the RVM on ARH could be one way to assess its causal role.

**Table 7.2** Summary of differences in levels of endocannabinoids and related lipids in the presence or absence of noxious stimuli in WKY and SD rats

Side	Brain region	ARH	Anxiety	Nociception in SD	Nociception in WKY
		WKY-Form vs SD-Form	WKY-Sal vs SD-Sal	SD-Form vs SD-Sal	WKY-Form vs WKY-Sal
left	dIPAG				
right	dIPAG	↑ 2-AG			
left	vIPAG	↑↑↑ all but AEA	↑↑↑ all but AEA		
right	vIPAG	↑ 2-AG	↑↑↑ all		
left	IPAG	↑↑ 2-AG, PEA	↑ 2-AG		
right	IPAG	↑↑↑ all but AEA	↑ 2-AG	↓↓ all but 2-AG	
left	BLA				
right	BLA	↑ PEA			
	RVM	↓↓↓ all	↑↑ PEA, OEA	↑↑↑ all but AEA	↓↓ OEA and PEA
left	vlHipp	↓ 2-AG		↑ 2-AG	
right	vlHipp				↑↑↑↑ all
left	dlHipp	↓ 2-AG	↓ 2-AG		
right	dlHipp		↓ 2-AG		
left	Ins				
right	Ins				
	PFC		↓ OEA	↓ PEA, OEA	

SD, Sprague Dawley; WKY, Wistar Kyoto; Saline; Form, Formalin; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; PEA, N-palmitoyl ethanolamide; OEA, N-oleoyl ethanolamide.

It appears that different forms of stress/fear/anxiety engage neural substrates differentially to significantly affect pain responding. At the behavioural level, high intensity stress or fear results in analgesia while long term stress/anxiety of a more moderate intensity results in hyperalgesia. These different forms of aversion might mobilise the endocannabinoid system differentially. Our data suggest that enhanced endocannabinoid signalling favours FCA and decreased endocannabinoid signalling might be responsible for ARH. These results suggest that opposing endocannabinoid mechanisms might mediate or facilitate FCA and ARH. While FCA appears to be the result of enhanced endocannabinoid activity in regions such as the dIPAG, ARH appears to be the result of, at least in part, diminished endocannabinoid activity in regions such as the RVM. Future work investigating the role of the endocannabinoid system in discrete brain

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regions in both FCA and ARH is needed. Our results suggest that while decreased *zif268* expression in the DHSC was associated with endocannabinoid mediated FCA, increased *zif268* in the RVM was associated with ARH, indicating a diverging response at the molecular level.

I believe further work is needed to improve understanding of this intricate interaction between pain and anxiety/fear and the role the endocannabinoid system plays in such an interaction. This work could be broadly classified as preclinical and clinical and should involve the following:

1. Further identification of neural substrates involved in endocannabinoid-mediated emotional modulation of pain. Alongside this, determination of novel genes/signal transduction molecules employing techniques such as microarray and proteomics is crucial. Such identification of molecular correlates significantly improves our understanding of the intracellular mechanisms underpinning the mechanism of pain and anxiety and their interaction and also could provide us with a possible therapeutic target for treatment of pain, anxiety or their co-morbidity.
2. Thus far, even though the role of the endocannabinoid system in emotional modulation of pain has been demonstrated in animal models, it hasn't been shown in human models. Demonstration of such a role in humans would ensure translatability of the principle that has been shown in the pre-clinical arena. Furthermore, using recent technologies such as fMRI, it is possible to determine key neural substrates involved in emotional modulation of pain. Even though it is not usually possible to collect brain/spinal cord samples in humans, plasma and cerebrospinal fluid samples could be analysed for measurement of endocannabinoids and related lipids. However, caution should be exercised when interpreting these results as it is not clear how well such measurements correlate with neuronal activity in the brain. Finally, examining post-mortem brain samples from patients who suffered from co-morbid pain and anxiety disorders, for example, could be one way to further improve our understanding of the pathology.

### ***References***

In conclusion, the work presented in this thesis represents the results of my work which have added to the body of knowledge in the fields of pain and fear/anxiety. These data advance our understanding of neurochemical and molecular alterations associated with pain, fear/anxiety and their interaction, and provide a solid foundation for future studies. The studies presented aid in the elucidation of the fundamental physiology of pain and fear and could potentially lead to new therapies for pain- and stress-related disorders.

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